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Nutrient Limitation of Phytoplankton in Helsinki Coastal Region

Helsinki Metropolia University of Applied Sciences

Bachelor of Engineering

Environmental Engineering

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May 2014

Helsinki Metropolia University of Applied Sciences

Author(s) Title Number of Pages Date	Bipin Dulal Nutrient Limitation of Phytoplankton in Helsinki Coastal Region 60 pages + 14 pages appendix May 2014
Degree	Bachelor of engineering
Degree Programme	Environmental Engineering
Specialisation option	Waste and Water treatment technology
Instructor(s)	Jukka Seppälä, Head of Marine Research Unit, SYKE. Veli-Matti Taavitsainen, Principal Lecturer, Helsinki Metropolia UAS

Bioassay factorial design experiments were carried out to study the limiting nutrient in Helsinki coastal area during the summer of 2013. The study also focused on finding out cost effective methods for modelling the phytoplankton responses and for identifying the particles and determining their density.

Nutrients (80 µg NH_4-N l^{-1} and 20 µg PO_4-P l^{-1}) were added to the 1 l water samples as per the 2^2 factorial design and chlorophyll concentration and chlorophyll fluorescence were measured regularly for 4 days. Sample bottles as well as the measurements were replicated. Polynomial regression method was used to analyse the time series data obtained from both methods, and significant coefficients were chosen on the basis of their *p* value than with normal score plots. The sample water of Day 0 and Day 3 were analysed with FlowCam and a microscope to identify the particles and to measure their density.

Chlorophyll concentration and chlorophyll fluorescence were measured separately to see the phytoplankton response to the added nutrients. The models obtained from the two different methods showed nitrogen as the limiting nutrient in Helsinki sea area. Chlorophyll fluorescence method was proposed for studying limiting nutrient in regular monitoring processes as it was found to give similar results faster and with lower cost than the chlorophyll concentration method. Taxonomical identification and particle density measurement were easier and faster with the FlowCam device than with the microscope.

Throughout the summer of 2013, nitrogen behaved as a limiting nutrient in Helsinki Sea area. Only four sampling stations within the range of 2 km from the main outlet of Viikinmäki Wastewater treatment plant were used. A few more sampling stations further from the outlet can be added in future monitoring program to study the co-limitation behaviour of nutrients or phosphorus domination.

Keywords	nutrient limitation, eutrophication, phytoplankton, polynomial regression modelling, Helsinki coastal region

Acknowledgement

This experiment was conducted by Environmental Centre of City of Helsinki in Marine Research Centre as a part of a regular monitoring program.

I am indebted to Seppo Kaitala, Leading Research Scientist, Ph.D, Finnish Environment Institute (SYKE), for giving me an opportunity to work as a research trainee in Marine Research Centre. I would like to thank Jukka Seppälä, head of the Unit of Marine Ecological Laboratory, and to Timo Tamminen for endorsing trust on me and enrolling me in the Nutrient limitation monitoring program. My special gratitude goes for Jukka Seppälä for his selfless support, energy and time in supervising me throughout the project. Jukka's friendly behaviour, generous help, encouragement and motivations are something which I will remember forever. I cannot forget thanking Emil Vahtera, researcher from City of Helsinki Environmental Centre, for regularly providing me all the information needed during the experiments.

Statistical data analysis and modelling was a challenging part of this project, and without the assistance of Veli-Matti Taavitsainen, it would have been a lot more difficult and time consuming task. I sincerely express my gratitude to Dr. Taavitsainen for his great support in supervising me in the mathematical part of this thesis. I am also thankful to Dr. Minna Paananen-Porkka for her academic assistances with the English language and thesis structure and to Kaj Lindedahl for his encouragement. I have enjoyed my four years of study in Helsinki Metropolia UAS in Leiritie, and I also wish to thank every teacher and staff of the degree program in Environmental engineering.

Finally, I would like to thank my parents, Ganga Dulal and Tulsi Dulal, for being with me in all my difficult times. I want to dedicate this thesis to them.

Thank you
Bipin Dulal
Helsinki, Finland.

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1 Introduction

Nutrients are essential for producers in any ecosystem. In aquatic ecosystems, phytoplankton such as dinoflagellates and diatoms are the main producers and important part of aquatic food chain. Phytoplankton requires various nutrients, sunlight, and favourable temperature to grow and to be available for higher animals who feed on them. Presence of essential nutrients in water under favourable condition can lead to rapid growth of phytoplankton, and absence of any essential nutrient can decrease the growth of phytoplankton. The increase and decrease in phytoplankton biomass plays a major role in altering the balance state of aquatic ecosystem.

Nutrients get into water bodies through various water and air mediums. Inorganic phosphorus and nitrogen are added mainly from the agricultural and forest runoffs, and from point sources such as treated or untreated waste water. The inputs of various nutrients into the water bodies from different sources cannot be uniform, and this makes water limited to certain nutrients. Uniform loading of essential nutrients can lead to severe eutrophication, but lack of any one essential nutrient in the receiving water can make the water limited by that particular nutrient. It is very essential to find out the limiting nutrient in any water body as it gives us some clues on predicting the future of the receiving waters. Absence or lack of any nutrient limits phytoplankton growth which again limits the growth of higher aquatic animals that feed on phytoplankton, whereas supply of the limiting nutrient can again lead to algae blooms. Such algae blooms lead to severe aquatic conditions, for example, decrease in dissolved oxygen, change in colour and smell of water and extinction of many species due to unfavourable condition.

Helsinki coastal region is loaded with nutrients from various airborne and waterborne mediums. Waste water from Viikinmäki waste water treatment plant is one of the main point source, which constantly loads a huge amount of nutrients into the coastal area of Helsinki. The presence of excessive nutrients in the sea water usually leads to algae blooms during spring and summer. Massive algae blooms make the sea water less attractive for recreational activities and also disturb the balance of the aquatic ecosystem.

Regular monitoring processes are inevitable in constantly observing the sea life situation. Andersen and Tamminen (2007) have monitored six different places in coastal areas of Finland, including Helsinki in 1993-1994, and have reported Helsinki coastal areas to be nitrogen limited. Taking Tamminen and Andersen's results into consideration, environmental body of city of Helsinki conducted similar bioassay experiments (mentioned in this thesis) in the summer of 2013 to find out the limiting nutrient and aquatic ecological state in the Helsinki sea area.

Although the total discharge from the wastewater treatment plant seems to be below the target level, a regular monitoring process is needed to study eutrophication behaviour in the areas, and to avoid any unfavourable condition in future due to degradation of the coastal water from the treatment plants discharges. This monitoring programme was initiated by the environmental centre of the City of Helsinki and Marine Research laboratory of Finnish Environment Institute to reveal the effect of discharging treated waste waters into the outer archipelago of Helsinki and also on the phytoplankton community. The aim is to evaluate the role of the effluent from treatment plant on the nutrient limitation. The monitoring process measures various parameters, such as identification of the limiting nutrient, nutrient concentration, the size distribution of cells, physiology of phytoplankton and any changes in their community composition. According to the environmental centre of City of Helsinki, this monitoring program will be incorporated as an obligatory program in the future for monitoring the coastal areas of Helsinki.

This thesis has four major objectives. First objective of this thesis is to find out the limiting nutrient, either nitrogen or phosphorus, in the coastal areas of Helsinki, and to compare it with a similar experiment conducted in 1993-1994 by Tom Andersen and Timo Tamminen (Andersen and Tamminen, 2007), which has shown nitrogen as the limiting nutrient in the Helsinki coastal areas. Similarly, regular monitoring process requires large number of samplings, experiments and analyses. Such processes can be expensive when they are done in large scale and thus cost optimization is important. The second objective is to compare two different methods of measuring the phytoplankton responses to added nutrients (chlorophyll-a concentration and chlorophyll fluorescence), and to find out the best results with minimum costs in conducting the two methods. The third objective is to compare the two statistical methods, *p* value method with normal score plot method, and find the better one of these, for modelling the phytoplankton growth. The fourth objective is to compare two different ways of conducting

taxonomical classification and identification of phytoplankton and to choose the most reliable and faster one.

Factorial design bioassay experiment on natural population is one of the best ways of determining nutrient limitation of any water bodies (Andersen and Tamminen, 2007 have done it in the Baltic Sea in 1993-1994 and Seppälä et al., 1998 have done it in Gulf of Riga). All the fourteen experiments conducted during the summer of 2013 have clearly shown nitrogen as the limiting nutrient in the Helsinki sea area. Added phosphorus has not shown any individual effect, and it is significant only in presence of nitrogen, which means it is not a limiting nutrient in this area. The Chlorophyll fluorescence method was found to be much faster and cheaper than the chlorophyll concentration method in determining the limiting nutrient in a regular monitoring method where sampling frequency is high. Analysing many samples for taxonomical identification and particle density was easier and faster with the FlowCam device than with a microscope. Normal score plot of the coefficients was based on the rule of thumb of normality, and choosing the significant coefficients was tedious and time consuming, whereas the *p* value regression summary table was easily generated by R-software and was used for selecting the significant coefficients during model plots.

This thesis has six different sections where the topics are explained in detail, making it easier for the reader with no or minimum knowledge in nutrient limitation and statistical data analysis procedures, to understand clearly every processes and findings. The first section is the general introduction of the thesis that gives brief information on the need for experiments, some of the previous similar researches and also on the objectives and main findings of this thesis. The second section explains the theoretical background needed in understanding the objectives, methodology and results of the experiments. The third section explains the different procedures for conducting the experiments. The fourth section is the result section which shows the important findings of the experiments, and finally the fifth and sixth section discusses and concludes the main findings of the results, respectively. Materials used in the experiments, statistical procedures for data analysis and R-script for model plot are included in Appendices.

2 Background

2.1 Eutrophication and phytoplankton

Eutrophication derives from Greek words '*eu'* meaning 'well' and '*trope*' meaning 'nourishment'. In aquatic system, eutrophication means enriching or nourishing the receiving waters with excessive nutrients, which leads to accelerated growth of phytoplankton and vegetation and decrease the quality of the ecosystem. This term is commonly used for the inputs of plant nutrients, such as nitrogen and phosphorus, into waters originating from various anthropogenic activities like agriculture and sewage treatment (Andersen et. al. 2006).The European Commission (EC), Urban Waste Water Treatment (UWWT) Directive defines eutrophication as 'the enrichment of water by nutrients, especially nitrogen and/or phosphorus, causing an accelerated growth of algae, and higher forms of plant life to produce an undesirable disturbance to the balance of organisms present in the water and to the quality of water concerned' (EC 1991). Eutrophication causes various environmental problems and it is a direct threat to the aquatic ecosystem.

Phytoplankton derives from Greek work *phyto* meaning plant and *plankton* meaning wanderer, thus they are the plant wanders in the water. Ocean current is the means of transportation for non-motile phytoplankton (USGS, 2001), while some flagellated species can swim with flagella. They are the main primary producers and very essential part of aquatic ecosystem. They play a vital role in maintaining the balance of the ecosystem as they are the first link in the marine food cycle. They provide food for zooplankton, mussels, oysters and small fishes and these animals in turn are food for bigger animals. Reproduction in phytoplankton is by cell divisions, and these result in huge accumulation of biomasses of phytoplankton, which become visible and are known as algae blooms. When algae die after algae blooms, decaying algae use dissolved oxygen, sometimes increase toxins in water, and may clog fish gills that can result in fish kill. Marine bio-toxins can harm human during fishing or while harvesting aquatic organisms. Bivalve shellfish and fishes that consume such toxic phytoplankton carry the toxins, and this can be transferred to the different level of consumers in the food chain.

2.2 Eutrophication as an environmental problem

Eutrophication leads to increase in algal blooms, decrease in dissolve oxygen concentration, changes in zoo benthos and fish communities, decrease in transparency and increase in bad smell making it unsuitable for recreational purposes (Bonsdorff et al., 1997). These environmental problems make the water bodies unsuitable for various aquatic animals. Some of the major environmental problems caused by eutrophication are explained below.

2.2.1 Harmful Algal Blooms (HABs)

Nutrients are necessary in aquatic life, but excessive nutrient loading can stimulate the unnecessary algae growth. The rapid growth of algae and its biomass accumulation in water bodies is known as algae blooms. Abundance algae in water have harmful as well as toxic effects on the aquatic ecosystem. More than 300 species of algae causes harmful effects, and about 80 species produce toxins, which can reach to humans through aquatic food web. Non-toxic algae can be harmful if the biomass production is rapid and high. Harmful algae blooms (HABs) are usually caused by microscopic phytoplankton which size ranges from 10 µm to 100 µm. These harmful species are found in most of the microalgae groups of diatoms, dinoflagellates, haptophytes and raphidophytes (Uronen P, 2007).

One of the major algae blooms is the cyanobacteria blooms (blue green algae bloom). These harmful blue-green algae blooms are also known as CyanoHABs (Cyanobacteria Harmful Algae Blooms). Water bodies rich in nutrients and with long residence time, water temperature of about 20˚C, calm surface waters, and absence of vertical mixing of water (persistent vertical stratification) are very susceptible to high CyanoHABs. Cyanobacteria blooms can be seasonal persisting only in summer and last till autumn, or can be persistent throughout the year (Havens, K.E., 2008).

Harmful algae in the Baltic Sea can be found in both marine and fresh water as the salinity differs in different sub basins of the Baltic Sea. *Nodularia spumigena*, *Aphanizomenon* species and *Anabaena* species are the filamentous cyanobacteria that form dense blooms on the surface of the Baltic Sea during late summer. Microalgae such as Dinoflagellates, *Dinophysis and* haptophytes *Prymnesium parvan* and *Chrysochromulina,* are the harmful species found around the world. *Chrysochromulina spp.* caused massive blooms in Skagerrak-Kattegat, a sub-basin of the Baltic Sea in the late 1980s.

Toxins produced by the haptophytes affected the zooplanktons and fishes (Uronen P, 2007), and this can affect humans while consuming these fishes (EC, 2002).

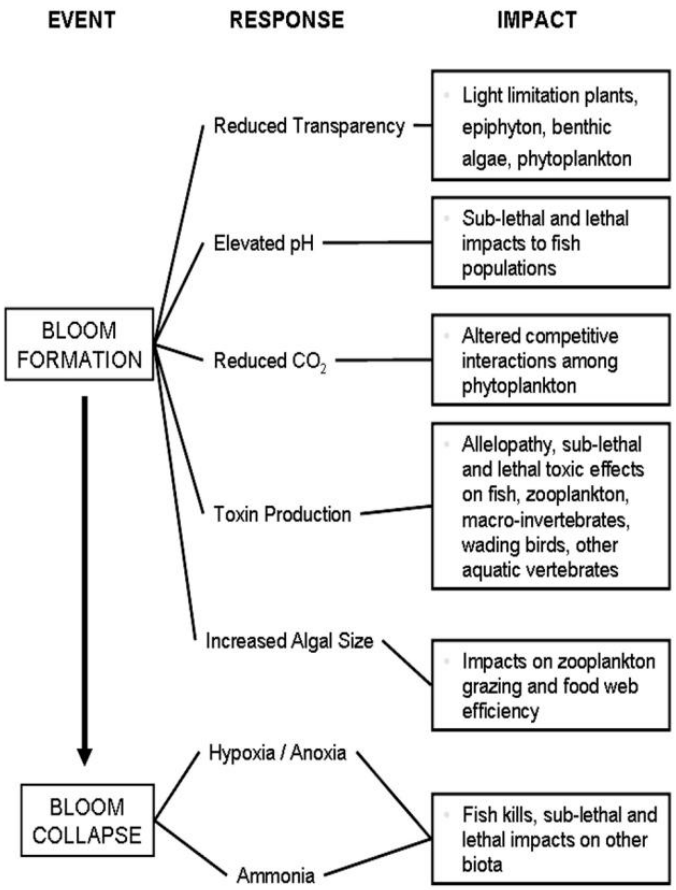


Figure 1. Summary of the ecological responses and impacts associated with blooms of cyanobacteria in lakes, rivers and estuaries. (Picture from (Havens, K.E., 2008). It shows the effects during algal blooms and after its collapse. Bloom formation has several negative responses for example it reduces the transparency of water, increases water alkalinity, produces toxins and kill fishes.

2.2.2 Oxygen depletion

Eutrophication is one of the major causes of oxygen depletion in water bodies. The nutrient enriched waters accelerate biological activities such as excessive growth of phytoplankton, cyanobacteria bloom and aquatic vegetation under suitable temperature and light conditions, and consume the available oxygen. Similarly, below the surface layer, the available oxygen is used to decompose the organic matter (usually the remains of algal bloom, zooplankton fecal pellets, dead plants and animals) by bacteria. The rate of depletion of oxygen to decompose the organic matter is much higher than the rate of production and resupply from the surface waters. This can result in hypoxic

or anoxic situation and may lead to death of various aquatic species, forced migration of animals to oxygen rich areas, reduction in habitat of species and their extinction, changes in aquatic food cycles and formation of dead zones (low-oxygen areas).

Prolonged stratification (formation of layers of water) reduces the circulation of nutrients and oxygen within the system. Lack of mixing of water at various levels can result in hypoxia (insufficient oxygen) and it affects the biological activities of the system. Animals capable of moving (crabs, fishes and shrimps) evacuates the area when the oxygen level falls below critical values (2 to 3 mg/l) whereas the animals living in the seabed, which are resistant to low oxygen level experience stress due to lack of oxygen, and may eventually die if the oxygen level falls further down to 1 mg/l or 0 mg/l (anoxic) (NOAA, 1998).

Excessive nutrients in water favor greedy and fast-growing microalgae and plants, and this creates imbalance in the aquatic food web, which leads to the accumulation of an abundance amount of phytoplankton in various strata of water bodies. High nutrients inputs have direct effects at the top layer of water bodies with an increase in phytoplankton biomass, and have indirect effects at the bottom layer of the water bodies with a low dissolved oxygen level and change in flora and fauna. Although in light, aquatic plants produce oxygen, but in darkness it is used by all animals and plants for respiration, and in case of severe biomass accumulation, the organic matter at the bottom of water bodies consume the available oxygen for oxidation. Oxygen present in sulphates (SO_4^{2-}) will be used by bacteria and release S^{2-} which uses available free oxygen to form sulphates, hence the available oxygen concentration for aquatic flora and fauna becomes insufficient. Limited oxygen availability inhibits the growth of zooplanktons, fishes and shellfishes. Algal growth, such as growth of microalgae, phytoplankton (diatoms, dinoflagellates, chlorophytes) and cyanobacteria enhance in sufficient presence of light, nutrients and temperature at the upper surface. Free floating microalgae and macrophytes at the surface prevent light transmission to the bottom, and oxygen depletion occurs as the submerged plants are unable to carry out photosynthesis. Hence, aerobic life at various layers below the surface of water decline due to lack of dissolved oxygen for respiration (EC 2002).

2.3 The Baltic Sea view

The Baltic Sea is one of the largest brackish-water basins in the world with catchment area of more than 1,700,000 km^2 and 85 million inhabitants. It is a shallow sea with an average depth of 52 m, with a volume of 21700 km^3 and a surface area of 415,200 km^2. The Baltic Sea has a long water residence time as it is inland sea and has limited water exchange with North Sea. High nutrient loading from agricultural sectors and long water residence time leads to slow discharge of the Baltic Sea water into North Sea, which makes it more sensitive to eutrophication as nutrients can remain for longer period of time. During summer most part of the Baltic Sea experience cyanobacteria blooms. Such blooms are potentially toxic to sea animals and humans. Increased phytoplankton biomasses make the water highly turbid and also decrease the marine recreational values (HELCOM 2009).

Nutrient inputs into the Baltic Sea have two main ways; waterborne inputs and airborne inputs. Among the main nutrients, nitrogen and phosphorus, 75 % of nitrogen get into the Baltic Sea through water medium and 25 % nitrogen through air medium. Among water medium, agriculture and forestry inputs (about 60 %), natural background sources (28%) and point sources (13 %) are the nitrogen sources. The airborne nitrogen input is due to deposition of direct atmospheric nitrogen through ship traffic and air emission from the catchment areas. The water medium for phosphorus inputs into the Baltic Sea is from agriculture and forest (50%), natural sources (25%) and point sources (25 %). The airborne phosphorus deposition in the Baltic Sea is not significant (1% - 5 %) (HELCOM, 2000).

2.3.1 Effluent from Viikinmäki WWTP

75% of phosphorus and nitrogen loading into the Baltic Sea is entirely from the water entering into it from inlands, where point sources such as treated waste water also play a major role in nutrient loading (HELCOM 2000). Nutrient loadings in Helsinki coastal region through point sources are the treated waste water from Suomenoja and Viikinmäki waste water treatment plants. Both of these treatment plants load a huge amount of nutrients into the coastal areas every year, thus it is necessary to see how the nutrients loading in these areas affect eutrophication (HSY 2011). In 2011, Suomenoja wastewater treatment plant on average discharged 100,000 m^3 of waste water daily and it loaded 571 tonnes of total nitrogen and 11 tonnes of total phosphorus into

coastal areas yearly. Similarly, Viikinmäki wastewater treatment plant on average treated 270,000 m^3 of wastewater daily and it loaded 473 tonnes of total nitrogen and 20 tonnes of total phosphorus into the coastal areas yearly. The estimated target of total nitrogen and total phosphorus loading into the Baltic Sea from the two treatment plants was 1100 tonnes of total nitrogen and 35 tonnes of total phosphorus per year, thus the treatment plants were successful in keeping their total discharges below the estimated targets in 2011 (HSY 2011).

Out of total nutrient loadings, 60% of the nitrogen is in inorganic form, i.e. in the form of nitrates (NO_3^-) and ammonium (NH_4^+), which are a bioavailable form for phytoplankton and 30 % of phosphorus is in the form of phosphates (PO_4^{3-}), which is also in a bioavailable form for the phytoplankton. This shows that every year Suomenoja WWTP loads 343 tonnes of inorganic nitrogen and 7 tonnes of phosphates and Viikinmäki WWTP loads 284 tonnes of inorganic nitrogen and 12 tonnes of phosphates into the coastal areas and are easily available for phytoplankton (HSY 2011). The other form of nitrogen and phosphorus gets converted into a bioavailable form slowly through various nutrient cycles.

3 Nutrient limitation

Phytoplankton consists of main three elements(C, N and P) at a certain ratio (Redfield 1958) and they require these essential elements along with some trace elements (Fe, Zn, Cd and Mn) (Cullen et al., 1999) for their growth. These essential nutrients are important for their growth and they get those from the water enriched with nutrients. Phytoplankton typically consume only the inorganic forms of nitrogen, i.e. nitrates, NO_3^- and ammonium, NH_4^+, (Dortch, Q. 1990) and inorganic forms of phosphorus, i.e. phosphates, PO_4^{3-} (Perry et al., 1981). Only nitrogen and phosphorus were chosen in these experiments as the limiting nutrients. (Wasmund et al., 2001) has explained that silicon is not a limiting nutrient for phytoplankton in the Baltic Sea. Nutrient enrichment experiment in the northern Baltic Sea with nitrogen, phosphorus, iron and EDTA (ethylenediaminetetraacetic acid) has shown that the impact of iron on total phytoplankton biomass is negligible (Vuorio et al., 2005).

If any of the essential nutrients is not present or if it is present in very low concentration, the phytoplankton growth decreases, but if that particular nutrient is supplied through any means to the phytoplankton, the phytoplankton growth rate increases

sharply. The nutrient that plays a major role in determining the growth of any organisms at its absence and presence is called a limiting nutrient (Beardall et al., 2001). In case of the Baltic Sea, the coastal region of Helsinki is nitrogen limited, the Bay of Bothnia is phosphorus limited whereas the Bothnian Sea and the Archipelago sea are both nitrogen and phosphorus limited (Andersen and Tamminen, 2007). There are many methods in determining the nutrient limitation in phytoplankton and some of the main methods are explained briefly below. The method used in this thesis is the nutrient enrichment experiment with natural phytoplankton population. Chlorophyll Fluorescence measurement of the natural phytoplankton population is also carried out as an alternative method.

3.1 Methods for Studying Nutrient limitation

There are different ways of determining the limiting nutrient in water bodies. The important ways are described briefly below.

3.1.1 Bioassays with Test Organisms

The bioassay experiment (an experiment with living organisms) can help to determine the limiting nutrient in a given water sample by measuring the growth of a test organism. Water sample from a particular site is filtered and then a test organism is inoculated in it. For freshwaters, organisms such as *Selenastrum capricornutum* (Miller et al., 1978) and for marine waters *Thalassiosira pseudonana* (Hayes et al., 1984) have been used. Nutrients are added in the water samples and then the growth of the test organism is measured. If particular nutrient is limited in the given water sample, then the growth of the test organism in the presence of that particular nutrient is significantly higher than the growth with the other nutrients as shown in Figure 2.

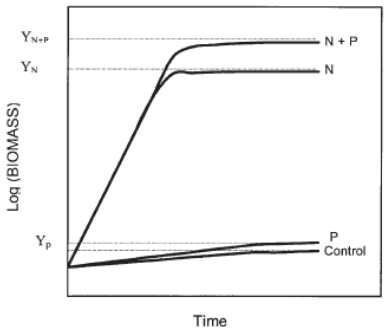


Figure 2. Bioassay growth of a nitrogen limited sample. Addition of phosphorus does not show any growth rate and behaves as a *control*. However, addition of nitrogen causes an increase in the growth rate. When N and P both are added, P becomes the limiting nutrient and yields the maximum growth ($Y_{N+P}>Y_N>Y_P$) (Beardall et al., 2001).

3.1.2 Enrichment experiment with natural populations

The enrichment experiment is carried with natural water samples. Unlike Bioassay experiment with test organisms, the natural phytoplankton present in the sample water is considered as an inoculum. Various nutrients are added in the natural water sample and various parameters such as carbon assimilation (Menzel and Ryther 1961), increase in biomass (Beardall et al., 2001), variable chlorophyll-a fluorescence parameters (Kolber et al. 1988; explained in fluorescence section) or chlorophyll concentration (Andersen and Tamminen, 2007) are measured for certain period of time that might range from some hours (Healey, 1979) to several weeks (Hecky and Kilham, 1988). If the productivity (14 C fixation or photosynthesis O_2 evolution) and growth (change in chlorophyll concentration or cell numbers) are not similar in the *control* and in the presence of a particular nutrient, then that nutrient which stimulates the growth or the production is considered as the limiting nutrient in the sampling site (Beardall et al., 2001). The experiment reported on in this thesis is a nutrient enrichment experiment with natural population where the biomass and chlorophyll fluorescence are measured to determine the limiting nutrient.

3.1.3 Elemental composition of phytoplankton and inorganic nutrients in water

The ratio of C, N and P present in phytoplankton and also in the water where they grow is used to determine the limiting nutrient. Redfield found that phytoplankton on average contain C, N and P in the ratio 106:16:1 (Redfield, 1958). If nutrients available for algae has a ratio of N: P significantly greater than 16, then the water is phosphorus limited, whereas if the ratio is smaller than 16, then the water is nitrogen limited (Beardall et al., 2001). However, the measurement of nutrients in phytoplankton to determine the limitation pattern is a complex process. Nitrogen and phosphorus are present in different forms in water and microalgae can only consume their bioavailable forms. Dissolved organic phosphorus and nitrogen are in complex form and are not available for phytoplankton. Not only microalgae but also some microorganisms consume and contain inorganic nutrients and thus analytical measurement of bioavailable phosphorus and nitrogen are complex processes and this does not guarantee that all measured phosphorus and nitrogen are potentially available for the phytoplankton (Bostrom et al., 1988). Elemental composition of phytoplankton measurement does not take into consideration the regeneration of nutrients, presence and input of organic and inorganic nutrients and the community structure of the autotrophs (organisms that produce their

own food) and heterotrophs (cannot produce their own food and rely on other organisms) in the water bodies (Beardall et al., 2001).

3.1.4 Chlorophyll-a fluorescence

Light energy absorbed by the light harvesting antennae of chlorophyll-a have 3 major competitive fates as shown in the Figure 3. It is emitted as heat and fluorescence, and used in photochemical work (photosynthesis within cell). The photochemical work is influenced by the presence of nutrients in the cells. Absence of limiting nutrient in the cell reduces the photochemical work and the surplus energy is liberated as fluorescence. High fluorescence emission from chlorophyll-a indicates that the photochemical work within cell has been highly reduced due to lack of limiting nutrients. Fluorescence can be measured by fluorometers and this is one of the tools for measuring the nutrient limitation in phytoplankton. The variable fluorescence is the most commonly used fluorescence parameter and it is expressed as F_v/F_m.

$$F_v / F_m = (F_m - F_0) / F_m$$

Where, F_0 is the minimum fluorescence (chemical work is maximum), F_m is the maximum fluorescence (chemical work is saturated or absent), and F_v is the difference between the maximum and minimum (F_m-F_0), (Beardall et al., 2001).

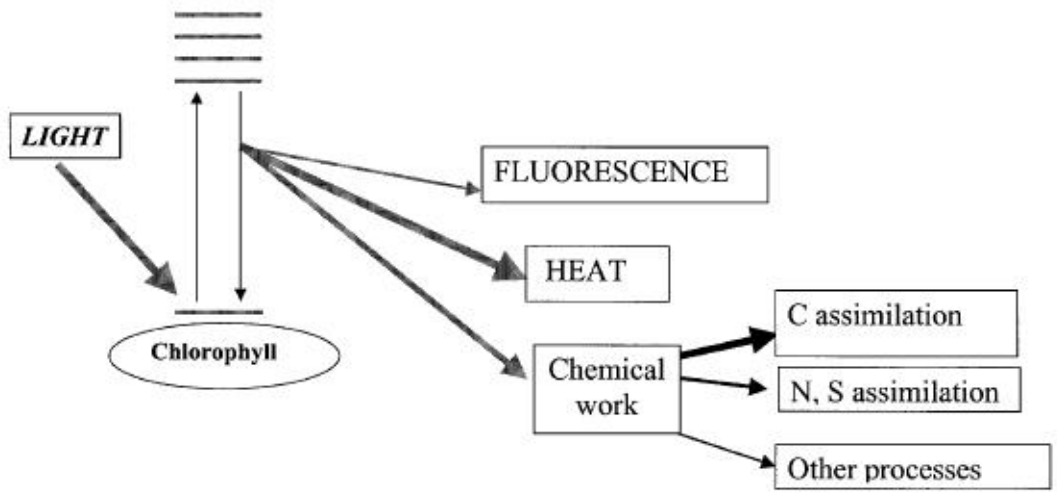


Figure 3. Distribution of light energy absorbed by chlorophyll in algal cells. Distribution of light energy absorbed by chlorophyll in algal cells.Excited chlorophyll molecule falls to the ground state and emits fluorescence, heat and performs chemical work with the released energy. More chemical work means less fluorescence and vice-versa so fluctuation in the assimilation processes can be reflected in fluorescence or heat output (Beardall et al., 2001).

The apparatus for harvesting photosynthetic light and energy transduction are rich in N and Fe, and P controls the cellular metabolism in the cell. Deficiency in these nutrients decreases the photochemical efficiency, *Fv/Fm*. This has been observed in N, P and Fe limitation in various algae (Kolber et al., 1988) and also diatoms show the changes in variable fluorescence in response to Si limitation and re-supply (Lippemeier et al., 1999). This photochemical efficiency (F_v/F_m) recovers if the limiting nutrient is re-supplied (Young et al., 1999) and can be used for determining the limiting nutrient in bioassay experiments on natural phytoplankton (Boyd et al., 1996). This thesis will show that the chlorophyll-a fluorescence method is faster and cheaper than the chlorophyll-a concentration method. However, in this thesis, only the value of F_0 from fluorescence dataset is used to model the phytoplankton response to the nutrient treatments and to classify the samples into various limitation classes.

3.2 Classes of Nutrient Limitation

While studying the limiting nutrient in a bioassay experiment, the phytoplankton can respond to either or both of the added nutrients, or may not respond to any of the added nutrients. Thus, there are seven different response possibilities that might occur as a result of the experiment. These possibilities are classified as classes of nutrient limitation. These classes are described below and illustrated in Figure 4.

No response (00)

Phytoplankton does not respond to any of the added nutrients. All the four treatments behave identically even their time course can be non-linear.

Primary P Limitation (P1)

If the water already has enough nitrogen in it, the addition of further N does not make any difference in phytoplankton response, so the *control* and the *N-alone* treatment behave in the same way**.** Addition of P in the *P-alone* treatment increases the phytoplankton response, but in the *PN-combined* treatment, the addition of P makes the water N limited and since N is available, the response further increases such that *P-alone* and *PN-combined* treatments behave differently.

Exclusive P limitation (XP)

If the water already has excess nitrogen in it, on addition of further N does not make any difference in the phytoplankton response, thus the *control* and the *N-alone* treat-

ment behave in the same way. Addition of P in the water increases the phytoplankton response but the *P-alone* and the *PN-combined* treatments behave in the same way.

Primary N limitation (N1)

If the water already has enough phosphorus in it, the addition of further P does not make any difference in the phytoplankton response, so the *control* and *P-alone* treatments behave in the same way**.** In addition of N in *N-alone* treatment, the phytoplankton response increases, but in the *PN-combined* treatment, the addition of N makes the water P limited and since P is already available, the response further increases such that the *N-alone* treatment and the *PN-combined* treatment behave differently.

Exclusive N limitation (XN)

If the water already has excess phosphorus in it, the addition of further P does not cause any difference between the phytoplankton responses in the *control* and in the *P-alone* treatment. An addition of N in water increases the phytoplankton response but the *N-alone* and *PN-combined* treatments show same response.

Primary Combined limitation (C1)

In this class, the all three added treatments have different effects from each other and also from the *control*. *PN-combined* has an effect on the phytoplankton growth, but *N-alone* and *P-alone* treatments also show growth different from each other.

Exclusive Combined limitation (XC)

The responses of the *control*, *N-alone* and *P-alone* treatments behave in the same way. However, the addition of P and N is so closely balanced that the *PN-combined* treatment is different from the other three similar treatments.

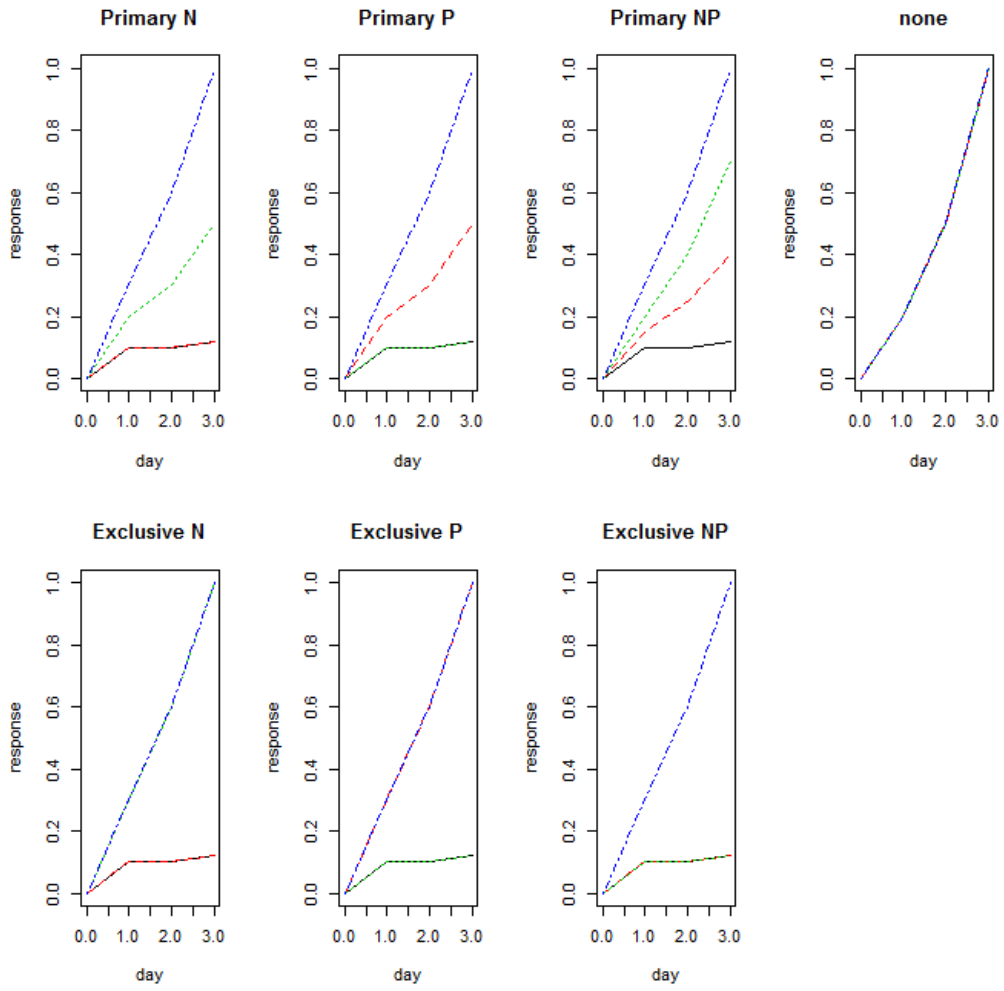


Figure 4. Seven different nutrient limitation classes as explained in Andersen and Tamminen, 2007. The *control* effect is represented by black dashed line, the *P-alone* by red dashed line, the *N-alone* by green dashed line, and the *PN-combined* by blue dashed lines.

4 Sampling, measurements, and experiment design and analysis

Waste water from the Helsinki Metropolitan area is collected and treated in the Viikinmäki waste water treatment plant. The effluent from the waste water is drained into the Baltic Sea and this is one of the main point source of nutrients in the Helsinki coastal region. The main outlet of the waste water treatment plant is 20 m deep under the Baltic Sea on the western slope of an underwater mound eight km away from the coast of Helsinki. The city of Helsinki decided to take four sampling points at certain distances from the effluent outlet as shown in the Figure 5. Table 1a mentions the co-ordinates of the sampling points and their distances from the effluent outlet, Table 1b

shows the experiment number and sampling frequency and Figure 5 shows the sampling points in a map.

Table 1. Location and sampling frequency of the sampling points. Table 1a shows the coordinates and distance of the sampling points from the Viikinmäki waste water outlet. Table 1b shows the sampling stations and sampling frequency with experiment number.

a)

	Lat	Long	Sampling Distance (m)
Outlet	60.090	24.918	0
125_a	60.091	24.914	250
125_b	60.092	24.910	500
125_c	60.094	24.902	1000
125	60.099	24.888	2000

b)

Week 2013	Station 125	Station 125_A	Station 125_B	Station 125_C
24	Exp_1			
26	Exp_2			
28	Exp_3			
30	Exp_4		Exp_5	
31	Exp_6	Exp_7	Exp_8	Exp_9
32	Exp_10			
35	Exp_11	Exp_12		
36	Exp_13	Exp_14		

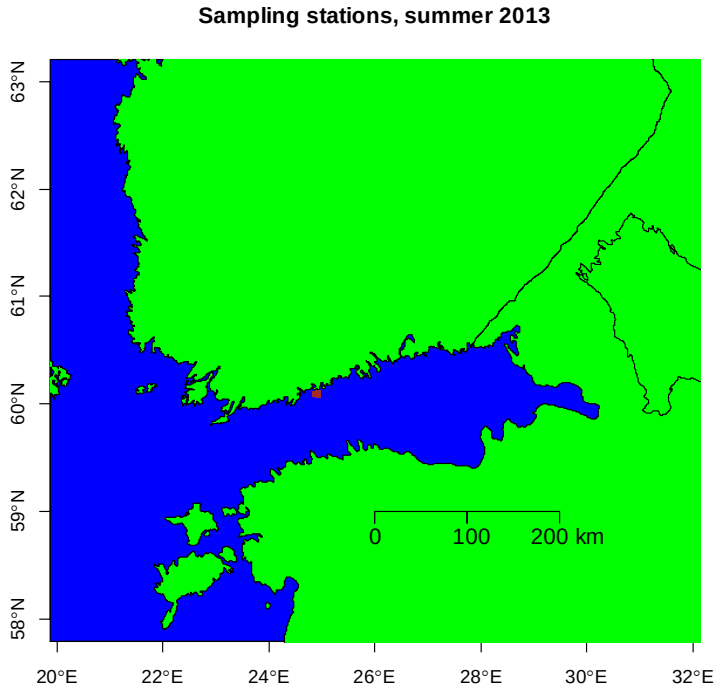


Figure 5. Location of the sampling stations. The sampling points are denoted by a single red dot as they are very close to each other. The map is plotted in R-software.

4.1 Water sampling

A motor boat was used to reach at various sampling points and sampling was done on the first workday, i.e. Monday, of every experiment week. During sampling, sample water was taken from different depths (0 to 10 m, 2l from each 2 meter depth) with the help of a water sampler (2.8 l) and temperature of water in each depth was measured with an attached thermometer in the sampler. The water from different depths was mixed and put into a sampling plastic container. The average temperature of the water was calculated and then the containers were transported to the Marine Research Laboratory. The thermostat water bath in the laboratory was labelled according to the name of the sampling point and the water temperature in the bath was maintained at the average temperature of the sampled water. Day length, with irradiance level of 100 µmol q m^{-2} s^{-1} in the water bath was maintained by using fluorescence tubes (OSRAM L 58W/965) and controlled by a timer. The water container was put in the water bath the whole night. It was ensured that there was no leakage and the water in water-bath was not overflowing on having the plastic container in it. The temperature of the water was adjusted from the temperature controller (LAUDA eco silver). The experiment started from the next day.

a) b)

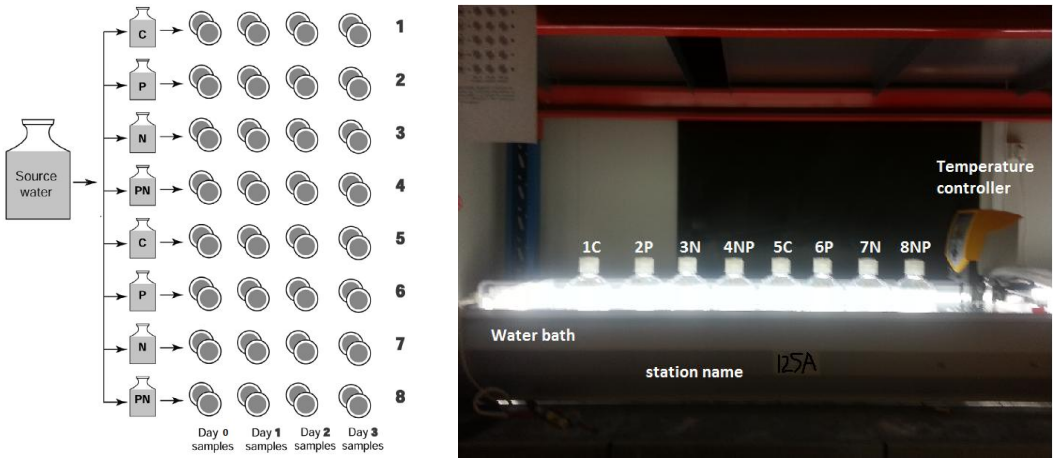


Figure 6. Experiment design and set up. Figure 6a is the basic experimental design (replicated 2^2 factorial designs) and sampling scheme for 4 repeated observations of chlorophyll-a response. 6b shows the actual picture of the waterbath in laboratory. It also shows the station name, temperature controller and labels of each bottle. 6b is an example of bottle arrangement on Day 0, on other days, the bottles are randomly arranged. Treatment codes: C = *control* (no nutrient addition), P = 1ml 0f 20 µg P l^{-1}, N = 1ml of 80 µg N l^{-1}, PN = *PN combine* addition (According to Tamminen and Andersen, 2007).

On the first day (Day 0), the plastic container (25 l) was taken out of the water bath and was thoroughly shaken. Eight identical round-bottles (Nalgene, each of 1 l or 2 l, 2 l bottles were used in Experiment 1 and Experiment 2; 1 l bottles were used in the remaining experiments), were filled with one litre sampled water (in Experiment 1 and Experiment 2, the volume of water used was 1.8 l and 1.6 l, respectively, because of 2 l bottles) with the help of measuring cylinder (1 l) and a funnel. The plastic container was shaken each time before drawing water from it. The round bottles were marked with water proof tapes and marker according to 2^2 factorial design, in an order, such that the first four bottles have C,P,N and PN, respectively; C = *control*, N = *N-alone*, P = *P-alone*, PN = *PN-combined*. The next four bottles were the replicated bottle samples and they were also labelled in the same way so that finally there were two bottles each of C, P, N and PN. Since there were two sets of bottles, in order to eliminate confusion, each bottle was given a separate number from 1 to 8 in an order 1C, 2P, 3N, 4PN, 5C, 6P, 7N, 8PN. These eight bottles were kept in the water bath and then nutrients were added into them according to their labels. 1ml of nitrogen-stock (conc. 80 µg/L, final conc. 80 mg/L) was added to the nitrogen bottles and 1ml of phosphorus stock (conc. 20 µg/L, final conc. 20 mg/L) was added to the phosphorus bottles.1 ml of N and 1 ml of P was added to the *PN-combined* bottles whereas no nutrient was added to the *controls*. After addition of nutrients, the bottles were thoroughly shaken and the bottle caps were tightened to ensure that no water would enter into the bottles if in case the bottle fell in the water bath.

Chlorophyll-a concentration measurement and chlorophyll fluorescence analysis in Aquapen (method explained in Section 4.2) were performed daily, starting from Day 0 till Day 3. Phytoplankton samples (for microscopic test) for all experiments and analysis in FlowCam (only for Station 125) were taken only on Day 0 and Day 3. However, this thesis contains only phytoplankton results of Experiment 6 and Experiment 7; and FlowCam results of Experiment 6.

4.2 Chlorophyll-a concentration measurement

Vacuum pressure (slightly below 200mbar) was created and tested using a pump. After completing the filtration set up, 1C (number 1, *control*) was taken and was thoroughly shaken. The cap of the bottle was replaced by a bottle top dispenser with its volume adjusted to 50 ml. Air bubbles were removed from the dispenser by pumping it once in a basin. After removing the air bubbles, 50 ml of water was filtered through a filtration

unit. The vacuum suction was opened to allow the water to pass through the glass fibre filter (25 mm diameter, nominal pore size 0.7µm, Whatman GF/F). After all the water has passed through the filter paper, the filter paper was taken out from the filtration unit and then put into its labelled glass vial (In this case, it is first day (Day 0), first bottle and *control* (1C)). The same process from the same bottle was repeated one more time to get the replicate. Finally, 10 ml of ethanol was poured into the glass vial and its cap was tightened firmly. The filtered paper was observed from outside of the glass vials to make sure that it has drowned in the ethanol. The same process was repeated for the other remaining seven bottles (2P, 3N, 4PN, 5C, 6P, 7N, 8PN). At the end of filtration on Day 0, there were sixteen glass vials filled with glass filter paper and ethanol. These 16 labelled glass vials were kept in deep freezer at -20℃. The eight water bottles were shaken only once before filtration and were kept in a random order in the water bath. The filtered water was collected in a container and this was emptied daily after the experiment.

a) b)

Figure 7. Filtration setup for chlorophyll measurement. Figure 7a shows the filtration unit for filtering chlorophyll-a. Figure 7b shows the Whatman GF/F Glass-microfiber filter paper after filtration. The circular dark area in the centre of filter paper is the filtration residue.

From second day (Day 1) till fourth day (Day 3), the same procedure mentioned above was repeated and the chlorophyll samples of every day were stored in deep freezer at -20℃. At the end of Day 3, sixty-four chlorophyll samples (16 samples per day *4 days) is obtained. Fourth day (Day 3) of all the experiment was usually Friday, and thus the chlorophyll concentration measurements was done on the next week (usually Monday). The same process was repeated for all other experiments for measuring chlorophyll concentration.

The chlorophyll concentration measurements of the first three weeks (24, 26, and 28) were done in Spectrofluorometer (JASCO FP-750), whereas the next five weeks (30, 31, 32, 35 and 36) measurements were done in Cary Eclipse Varian Fluorescence spectrophotometer. The measurement procedure of these two spectrophotometers slightly differed.

Before measuring the concentration of chlorophyll, all the chlorophyll samples were taken out from the deep freezer (-20℃) and then kept in a room for two to three hours to attain the room temperature. Before starting the analysis, the glass vials were roughly tested (with hand) to ensure that they had reached the room temperature and then chlorophyll concentration measurement was started.

Spectrophotometer JASCO FP-750 was calibrated with pure ethanol (chlorophyll-a concentration = 0 µg/l) and two standards (chlorophyll-a concentration = 0.0438 mg/l and 0.243 µg/l). The chlorophyll-a concentration was measured using excitation wavelength of 430 nm and emission wavelength of 668 nm with 10 nm slits. The filtered chlorophyll-a samples were transferred in to the JASCO through a hose.

Cary Eclipse Varian Fluorescence spectrophotometer was calibrated with six standards with concentration of 0 mg/l, 0.0092 mg/l, 0.0459 mg/l, 0.0917 mg/l, 0.1376 mg/l and 0.1824 mg/l. The excitation wavelength was 430 nm and emission wavelength was 670 nm with 5 nm silts. The filtered chlorophyll-a samples were transferred into the spectrophotometer with well plates. The chlorophyll-a concentration measurement was done for all the experiments.

4.3 Fluorescence measurement by Aquapen

Aquapen (Photon system instrument) is a sensitive, pocket sized FluoroPen fluorometer. It uses a cuvette and its measurement is quick, reliable and easily repeatable. It is equipped with blue and red LED emitter and delivers light intensities up to 3,000 µmol (photon).m-2.s-1 to measure the suspension. Blue excitation light (455nm) is used for measuring chlorophyll fluorescence from algae and red–orange excitation light (620 nm) is used for measuring phycobilins in cyanobacteria. It uses FluoroPen software for data presentation. Aquapen measures various parameters such as instantaneous chlorophyll fluorescence, chlorophyll fluorescence transient, light curve, quantum yield, and optical density from the given sample (PSI, 2013).

In this thesis project Aquapen was used to measure the fast chlorophyll-a fluorescence transient (OJIP), which is an important biophysical signal to measure the photosynthesis process of the phytoplankton that occurs in the treated water samples. The photosynthesis process in the sample is determined by the phytoplankton responses to the added nutrients and higher phytoplankton response gives higher photosynthetic value which shows the effects of added nutrients to the water. OJIP measures various parameters of chlorophyll fluorescence, but in this thesis project only the value of minimum fluorescence (F_0), which is the fluorescence intensity at 50 µs (microseconds) was analysed.

Before measuring the fluorescence, 10 ml of water from each treatment bottles was taken in separate glass vials and the glass vials were stored in dark for about 30 minutes. The OJIP mode in Aquapen was started. The glass vials were thoroughly shaken and then the water was transferred into quartz cuvettes (QS 10 mm) and all samples were then run through the Aquapen. The measuring was done with the blue excitation light at a wavelength of 450 nm as it measures the chlorophyll fluorescence. Replicates were taken for each measurement. Chlorophyll fluorescence measurement was done only for the first ten experiments.

4.4 Taxonomy and particle density measurement by FlowCam

FlowCam is a fluid imaging device that collects images and various other parameters of the particles present in the fluid passed through the device. It is equipped with optical, electronic and fluidal technologies for particle analysis. The fluidal system has an ultra-high precision computer-controlled syringe pump that pulls the sample fluid and flow it perpendicular to the optical path. The optical system captures the picture of the particle that flows through the flow cell in the fluidal system, perpendicular to it. The electronics are optional systems for capturing two fluorescences' information from the same particle (Fluid imaging technologies, 2013).

Before starting the particle imagine analysis in FlowCam, the treatment bottles were thoroughly shaken and about 50 ml of the water was poured into a glass beaker. The water was transferred into the FlowCam device by submerging the rubber hose of the syringe pump into the sample water. In the beginning, the water was allowed to move through the hose to remove all air bubbles present in the hose. After all air bubbles were removed, the computer program 'x' was opened and the laser light in the FlowCam was turned on. FlowCam captures the pictures of the particles (mainly phyto-

plankton) present in the water and measures the particle density. The FlowCam was run for one hour for each sample. FlowCam was used only for Station 125 in all experiments. The other stations' water was not analysed by FlowCam because their sampling was not regular and it took a minimum four and half hours on the Day 3 to complete analysing four different bottles of one station. However, the result of only Experiment 6 (Station 125, Week 31) is included in this thesis.

4.5 Taxonomy and particle density measurement by microscope

Microscopic test was carried out to find the different species of phytoplankton present in the treated water along with their size, volume and biomass. The test was conducted with a microscope (Leica DMI3000 B). About 100 ml of sample water was taken in brown bottles and were stored in a freezer at 4℃. 10 -12 drops of Lugol solution was added in the bottle to kill the phytoplankton present in the sample water and to stop its growth. Microscopic tests were done after few weeks, but the obtained results resemble the phytoplankton biomass at the time when the Lugol's solution inhibited the phytoplankton growth.

5 Results

The main goal of the experiments was to analyze nutrient limitation for the response of the phytoplankton to the nutrient treatments for different experiments. The obtained models showed which nutrient were more effective and which of the time effect; linear, quadratic or cubic was dominant in phytoplankton growth. Two different statistical methods were used to check the best method that gives more precisely the statistically significant coefficients. The better statistical method was used throughout the experiment to select the significant regression coefficients that fits the response model. However, the procedures for both methods are explained briefly in this section.

5.1 Selection of regression coefficients using p-statistics

The *p* values of the regression summary were used to select the significant coefficients. Following three criteria were used to examine how well the obtained model explained the phytoplankton response to the nutrient treatments.

1. The adjusted-R-squared value from the regression model summary table was used to determine how well the obtained model fit the phytoplankton responses.

2. Lack of fit *F* test was carried out to test if the obtained model suffered any lack of fit due to removal of non-significant coefficients.
3. Residual analysis (Normal probability plot of residuals) was performed to see normality in errors.

Chlorophyll-a result of the Experiment 1 (Station 125, Week 24) is taken as an example and explained in detail in this section. The explanation includes the summary of initial model, summary of selected model (obtained by removing the non-significant coefficients from the initial model) result of lack of fit *F* test and normal probability plots of residuals of the Experiment 1.

The figures of the fitted models of the chlorophyll-a data of all the experiments are present in this section. The regression output of the other thirteen experiments is not explained here in detail. The regression summary tables of the selected model are presented in Appendix 2A and the normal probability plots of residuals of the other thirteen experiments (except Experiment 1) are in Appendix 2B.

An orthogonal matrix was used for deriving linear, quadratic and cubic time effects. The (4 x 4)orthogonal matrix, the derivation of the equations for linear, quadratic and cubic time effects, the dimension check of the dataset and a detailed explanation of various steps before generating the models in R are given in Appendix 1B.

The mathematical equation of the model for the phytoplankton response to the nutrient treatment is

$$\begin{aligned} y = b_o + b_{10}P \quad + b_{20}N \quad + b_{120}P*N \\ + b_{11}P*t_1 + b_{21}N*t_1 + b_{121}P*N*t_1 \\ + b_{12}P*t_2 + b_{22}N*t_2 + b_{122}P*N*t_2 \\ + b_{13}P*t_3 + b_{23}N*t_3 + b_{123}P*N*t_3 \end{aligned}$$

where y is the phytoplankton response to the treatments (concentration of chlorophyll-a), P is phosphorus treatment; N is nitrogen treatment; t_1 is linear time effect; t_2 is quadratic time effect, t_3 is cubic time effect, b_o is the intercept and the other b's are the regression coefficients.

In R, the above mathematical equation is expressed as

```
> model <- lm(y~(P*N)+(ti+t2+t3)*(P*N))
```

The explanation of this equation in R and how it generates the sixteen coefficients are explained in Appendix 1B.

5.1.1 *F* test for overall regression

The hypothesis for the overall regression *F* test describes the null hypothesis as '*except intercept, all coefficients are zero'* and the alternative hypothesis as '*at least one coefficient is non-zero except the intercept'*.

Table 2. Summary table of R output of initial model of Experiment 1(Station 125, Week 24).

Exp_1					
Variables	Coefficients	Std. Error	t-value	p -value	
Intercept	4.190469	0.027693	151.317	2,00E-16	***
P	0.347344	0.027693	12.543	1.08e-09	***
N	0.757656	0.027693	27.359	7.30e-15	***
t1	0.256469	0.012385	20.708	5.59e-13	***
t2	-0.111406	0.027693	-4.023	0.000984	***
t3	0.102781	0.012385	8.299	3.43e-07	***
P:N	0.415781	0.027693	15.014	7.54e-11	***
P:t1	0.187844	0.012385	15.167	6.47e-11	***
N:t1	0.303906	0.012385	24.539	4.00e-14	***
P:t2	0.217969	0.027693	7.871	6.85e-07	***
N:t2	0.194531	0.027693	7.024	2.87e-06	***
P:t3	0.014906	0.012385	1.204	0.246254	
N:t3	-0.007656	0.012385	-0.618	0.545146	
P:N:t1	0.196531	0.012385	15.869	3.28e-11	***
P:N:t2	0.190156	0.027693	6.867	3.79e-06	***
P:N:t3	0.013219	0.012385	1.067	0.301661	

The Table 2 above shows the sixteen coefficients with their standard errors. The standard errors are the estimated standard deviations of the coefficients. The *t* value column is the ratio of the coefficients and the standard error. It is used for calculating the probability value (*p* value).The *p* value column gives the probability value of each coefficient and on the right of *p* value column are the stars that show the level of significance of the coefficients. Highly significant coefficients are denoted by three stars and their *p* value is almost zero. Statistical significance decreases with the decrease in number of stars but two stars and one star are still mathematically significant and are included in the model. The dot symbol '.' and empty ' ' shows that the coefficients are

not significant and are ignored in the model. The *F* statistic is the *F* test of the overall regression of the model. Residual standard error is 0.1567, which is the standard deviation of the residuals and degree of freedom is 16 and that denotes the difference between the number of chlorophyll-a responses and the number of coefficient used in the model. The residual standard error and degree of freedom from the summary table are used in lack of fit *F* test calculation. The obtained adjusted R squared value of 0.9893 states that the obtained model explains almost 99 percent of the data.

The *p* value of the overall regression *F* test is 3.625e-15, which is almost zero and lower than the standard significance level (0.05) and thus the null hypothesis is rejected and the alternative hypothesis of the overall regression *F* test is accepted, which means there is at least one coefficient, in addition to the intercept, that contributes significantly in the model. This is true as the above model has shown 13 significant coefficients and 3 non-significant coefficients in the summary table. The *p* values of these thirteen coefficients are lower than the standard significance level and their significance is also shown in the table by the stars. However, the *p* value of the coefficients P: t3, N: t3 and P: N: t3 are much higher than 0.05 and thus they are the non-significant coefficients. For these three coefficients, the null hypothesis is accepted that these coefficients are zero and then remove them from the initial model.

For each experiment, among the sixteen coefficients, only the significant coefficients are included in the model. After removing the non-significant coefficients, a new model is obtained with the significant coefficients, named as selected model. In selected model, the significant coefficients remain same but the value of the estimated standard errors, *t* value, *p* value of the coefficients, the F statistics and its *p* value, R-squared and the adjusted R-squared and the degree of freedom of the model changes on removing the non-significant coefficients from the initial model. The R-command for the final model of the Experiment 1 is shown below:

```
> model2 <- lm(y ~ P*N+(t1+t2+t3)*(P*N) - P:t3 - N:t3 - P:N:t3)
```

The summary of the selected model of the Experiment 1, Station 125 has thirteen significant coefficients (3 non-significant coefficients are removed from 16 coefficients). The *p* values of these 13 coefficients are much lower than the standard significance level and are significant. The adjusted R-squared value (0.9894) is slightly smaller than the R-squared (0.9935) and shows that the obtained model explains about 99 percent

of the data. The adjusted R-squared is accepted as it takes into consideration the degrees of freedom and is more reliable than the R-squared. (Neter et al.,1996, p 230-231)

5.1.2 Lack-of-fit *F* test

Another hypothesis test is carried out to see if there is any lack of fit in the selected model. The null hypothesis is defined as *'There is no lack of fit in our model'* and the alternative hypothesis as *'There is a lack of fit in our model'* (Montgomery. 2001). Lack of fit in a model suggests that the response behavior is more complicated than the model can explain.

Lack of fit *F* test is carried out to see how well our phytoplankton response model fits into the chlorophyll-a data. Lack of fit *F* test of Experiment 1(Station 125, Week 24) is obtained to be 0.99 with 3 degree of freedom. Its *p* value is 0.42 which is greater than the standard significance level (0.05), (0.4224965 > 0.05). Since, *p* value is greater than 0.05, the null hypothesis is not rejected and conclude that there is no significant lack of fit in the selected model of the Experiment 1.The calculation of lack of fit *F* test is explained in Appendix 1C and the results of lack of fit *F* tests of all experiments is given along with R regression summary table in Appendix 2A.

5.1.3 Normal probability plots of the residuals

The normal probability plot of the residuals of Experiment 1 in Figure 8 shows that the true errors of the model are normally distributed (Box and Draper, 2007. p.114). This supports that the obtained model is a good model. However, the sample number 12 in Figure 8 does not lie reasonably closer to the straight line which conveys that it is different from its replicate and do not fit in the predicted model. Response 12 is the Day1, *PN-combined* treatment (Bottle 4) and its replicate is response 16, Day 1, *PN-combined* treatment (Bottle 8). In Figure 9, the model plot shows that on Day 1, the Bottle 4 and Bottle 8 do not overlap and are a certain distance showing that the replicates deviate from each other. This deviation is also seen in the Bottle 4 and Bottle 8 in Day 2 and Day 3 in Figure 9. The normal plot of residuals in Figure 8 shows this deviation clearly as residuals of the replicated responses such as 12 and 16, 20 and 24(Day 2, Bottle 4 and Bottle 8), 28 and 32 (Day 3, Bottle 4 and Bottle 8), are also far from each other. In normal probability plots of the residuals of chlorophyll concentration of Experiment 3, Experiment 6 and Experiment 11(see Appendix 2B); there are strong

outliers and the residuals are not closer to the straight line which is due to huge difference between replicates. Since there are only two replicates, it is very difficult to predict which one of them is completely different from the actual value and thus a reliable conclusion cannot be drawn at this point. The normal probability plots of the residuals of chlorophyll-a concentration of all the experiments are shown in Appendix 2B.

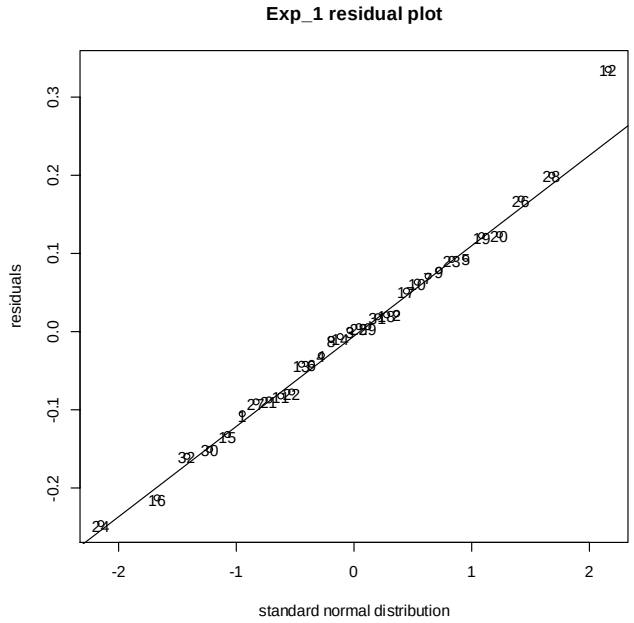


Figure 8. Normal Probability plot of the residuals of Experiment 1.

In the Figure 9, the numbers from 1 to 8 represent the treatment bottles. The four lines are the fitted models on the data. The model clearly shows that the *P-alone* treatment behaves in the same way as the *control,* which means the phytoplankton does not respond to the additional phosphorus. In *N-alone* treatment and in *PN-combined* treatment, the response of the phytoplankton is much higher than the *P-alone* treatment and the *control* treatment. This shows that the water is deficient in nitrogen and its addition stimulates the growth of the phytoplankton. The combined addition of the nutrients has shown the maximum response which means that phytoplankton responds to the added phosphorus only in the presence of sufficient nitrogen. The graph show sharp increase in the biomass in the combined addition of two nutrients, more than two folds as compared to *N- alone* treatment response. This is a clear example of primary nitrogen limitation class as the *PN-combined* treatment has the higher response than the *N-alone* treatment whereas the difference between the *control* and *P-alone* treatments is indistinguishable.

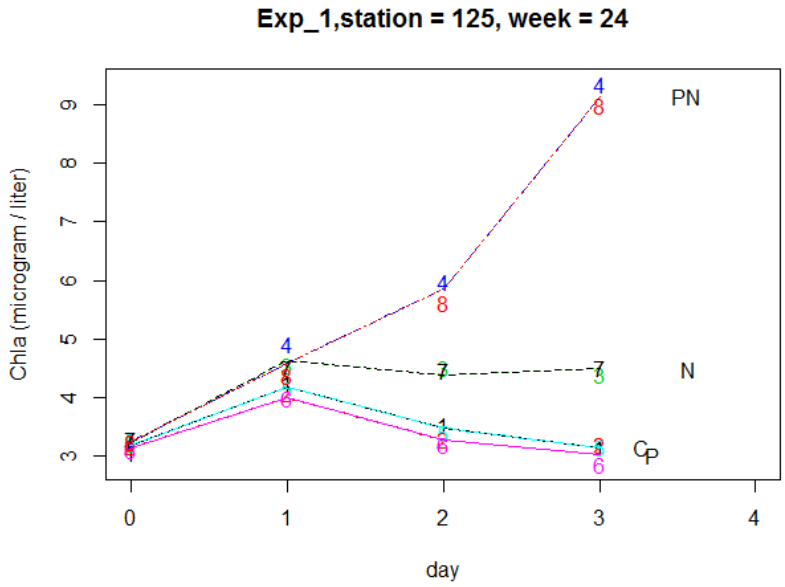


Figure 9. Fitted model on the chlorophyll-a data for Experiment 1. The numbers in the graphs represent the nutrient treatment bottles, the line are the fitted chlorophyll-a models.

5.2 Normal Score plot of the coefficients

Another way of generating the phytoplankton response model is with normal score plot method, either in R-software or in matlab (Seppälä et al., 1999). The normal score of the coefficients is plotted and the significant coefficients are selected by separating the non-significant coefficients visually on the basic of their standard deviation. The non-significant coefficients align themselves in a straight line whereas the significant coefficients remain as outliers (Box and Draper, 2007, pp.241). This method gives almost the same result given by *p* value method, but the selection of significant coefficients by *p* value is more precise and statistically more reliable than the normal score plots. The detailed explanation of the methods used by the vector method is explained in the Appendix 1E.

Figure 10 shows the normal score plot of the coefficients that is obtained in R or matlab windows. The y-axis is the score value of the normalized coefficients whereas the x-axis is the normal standard means ($\mu = 0$, $\sigma^2 = 1^2$). The non - significant coefficients group themselves in a straight line (within the zero mean and $\pm 3\sigma$), whereas the significant coefficients are outside this range and selection of the significant coefficients is done visually. The first click is taken at the left hand side of the fitted line and the second click at the right hand side to separate the non-significant coefficients. The coeffi-

cients that lie in-between the two clicks are the non-significant coefficients and the coefficients that lie outside the two clicks are the significant coefficients. The names of the significant coefficients can be seen on the graphs after completing the two clicks and their values can be obtained in a matrix form (4 x 4) by typing *bnp* (name given to the significant coefficients, for details see Appendix 1E) in R or in matlab window. After the clicks, the figure of fitted model on the chlorophyll-a response data is obtained as shown in Figure 11.

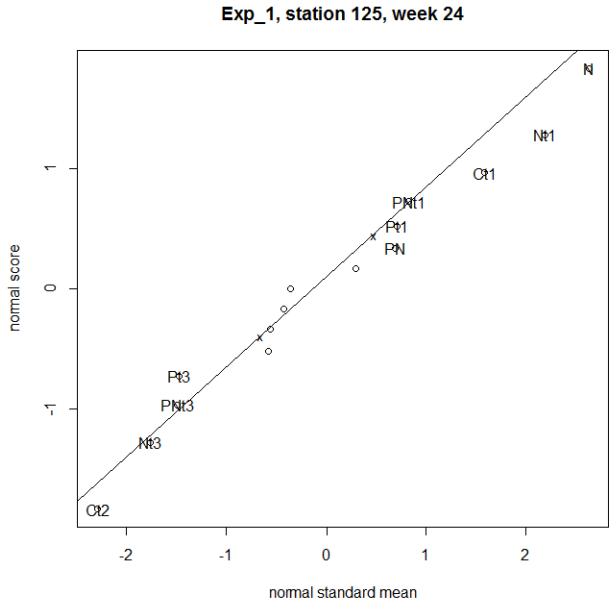


Figure 10. Normal score plots of the coefficients.

The symbol 'x' denotes the mouse clicks on the graph. The first click is at the left side and the second click is at the right side. The Figure 10 shows 10 coefficients as significant out of 16 coefficients. It assumes the remaining six non-significant coefficients as zero. The model obtained from these ten significant coefficients is shown in the Figure 11.

Table 3. Coefficients of the Experiment 1, by normal score plot. The rows in the above matrix are the treatments (C, P, N, PN) and the columns are the time effects (t0, t1, t2, t3).

	[,1]	[,2]	[,3]	[,4]
[1,]	4.190469	0.256469	-0.11141	0
[2,]	0	0.187844	0	0.014906
[3,]	0.757656	0.303906	0	-0.00766
[4,]	0.415781	0.196531	0	0.013219

The normal score plot gave only ten significant coefficients when the selection of significant coefficients was made by the author on the basis of the standard deviation of the coefficients. The zeroes in the Table 3 above are the non-significant coefficients. This almost gives the same result as given by the *p* value method. The phytoplankton response models obtained from normal score plots of coefficients (Figure 11) and from the *p* value method (Figure 9) differ slightly. This is due to the difference in the number of significant coefficients chosen by the two methods.

If the significant coefficients obtained from the p-statistics summary table is compared with the significant coefficients chosen by human eye based on ±3ϭ on the normal score figure, the difference is only in the number of significant coefficients (not in values as they are computed from the same response data). The *p* value method gives thirteen significant coefficients whereas normal score plot gives only ten significant coefficients.

The significant coefficients after regression from the data of Experiment 1 by the two different statistical methods did not match with each other. However, in some of the experiment they matched exactly with each other. In the normal score plot, the selection of coefficients is done visually on the basic of ±3σ and thus it has high probability of getting errors and the selection points might also differ from person to person whereas in *p* value method, the significant coefficients are chosen by R by comparing it with the standard significance level (0.05).

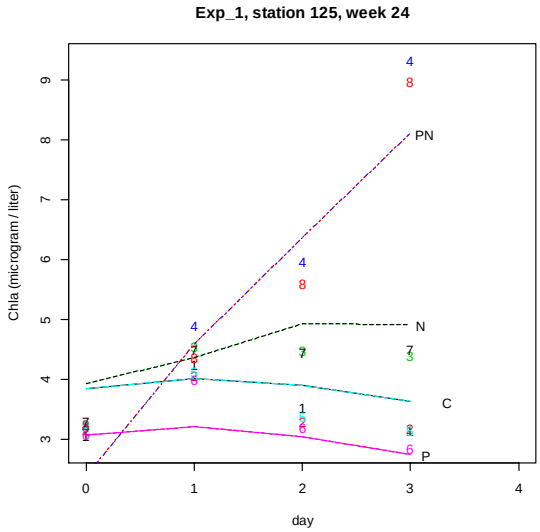
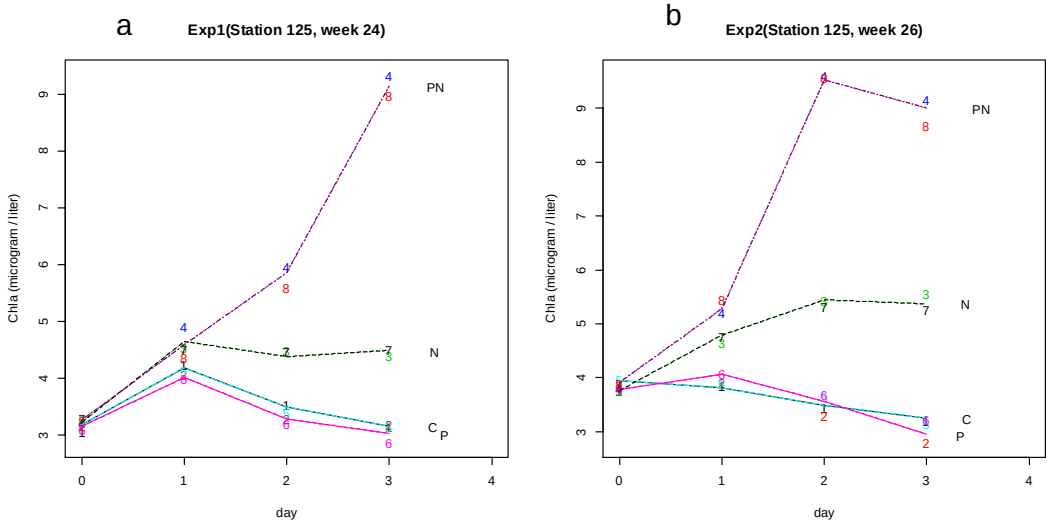


Figure 11. Fitted model of the phytoplankton response on the chlorophyll-a data for Experiment 1, Station 125, Week 24, obtained from the normal score plot of the coefficients.

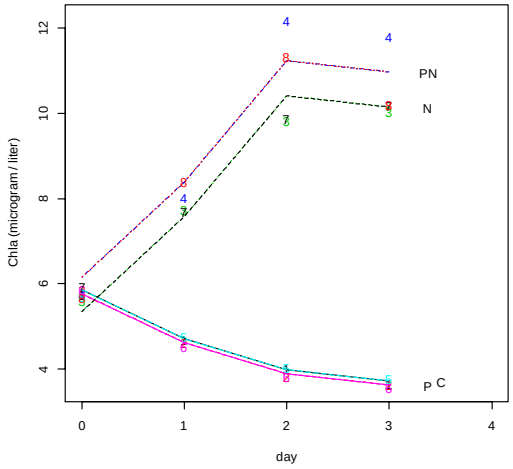
Probability value (*p* value) is not used to determine the significance of the coefficients in normal score plot. R-squared value of the normal score plot is nearly 0.86 which is much lower than the R-squared obtained from the selected model summary from the first method. However, in *p* statistics regression summary table, the probability-value of the coefficients determines the significance by comparing it with the standard significance level (0.05). This method is more statistically reliable, faster and easier in selecting the significant coefficients than the normal score plot method. On the basis of this result, further modeling of the phytoplankton responses of the remaining thirteen experiments was carried out with the *p* value method.

5.3 Chlorophyll-a concentration measurement results for the 14 experiments

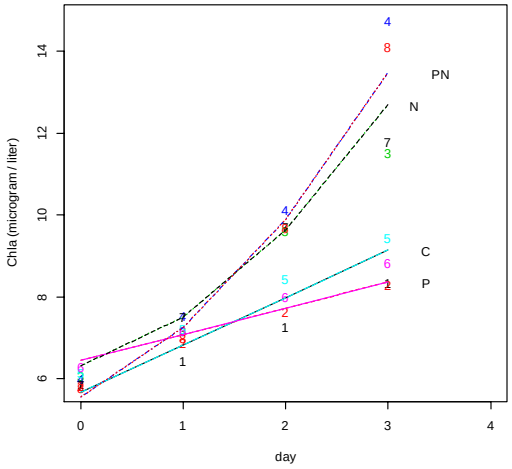
The final models of all chlorophyll-a concentration measurement methods were generated with *p* value method and are shown in Figure 12. The response models of all the experiments have clearly shown nitrogen as the limiting nutrient in the Helsinki coastal region.



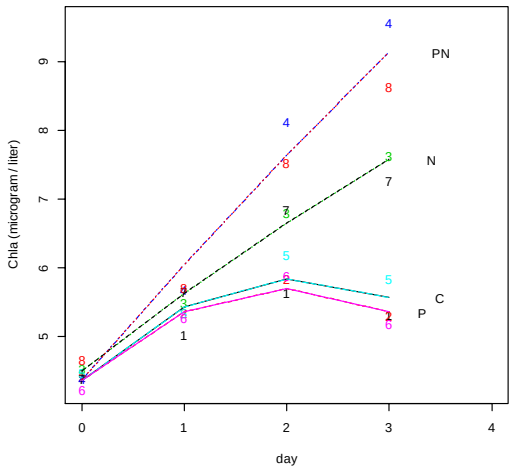
c Exp3(Station 125, week 28)



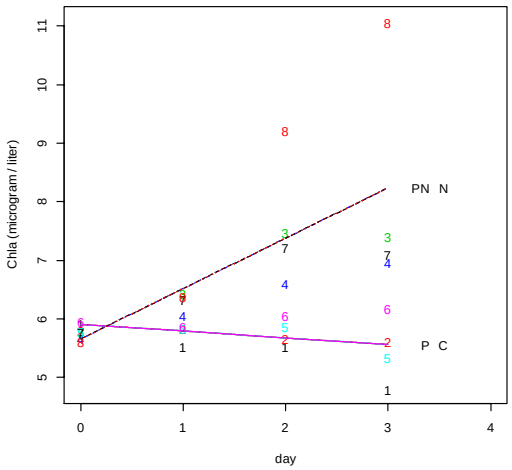
d Exp4(Station 125, week 30)



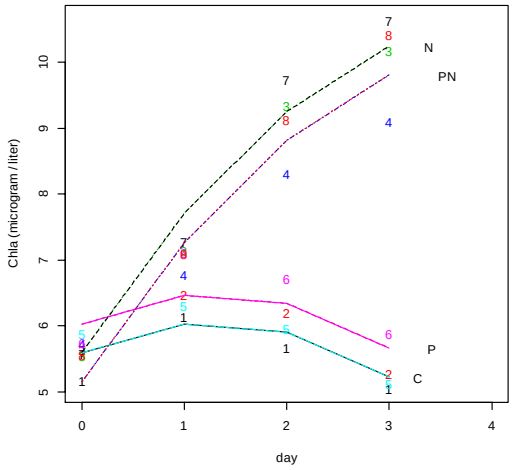
e Exp5(Station 125_B, week 30)



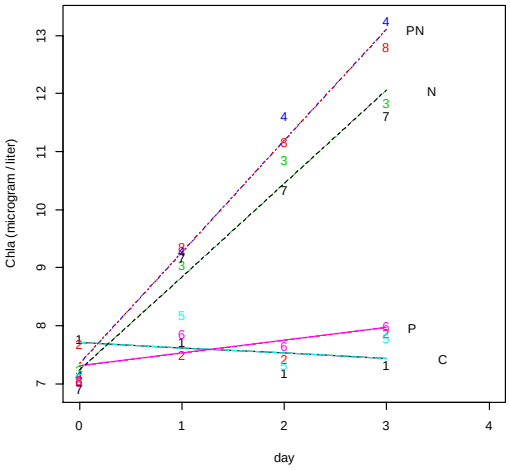
f Exp6(Station 125, week 31)



g Exp7(Station 125_A, week 31)



h Exp8(Station 125_B, week 31)



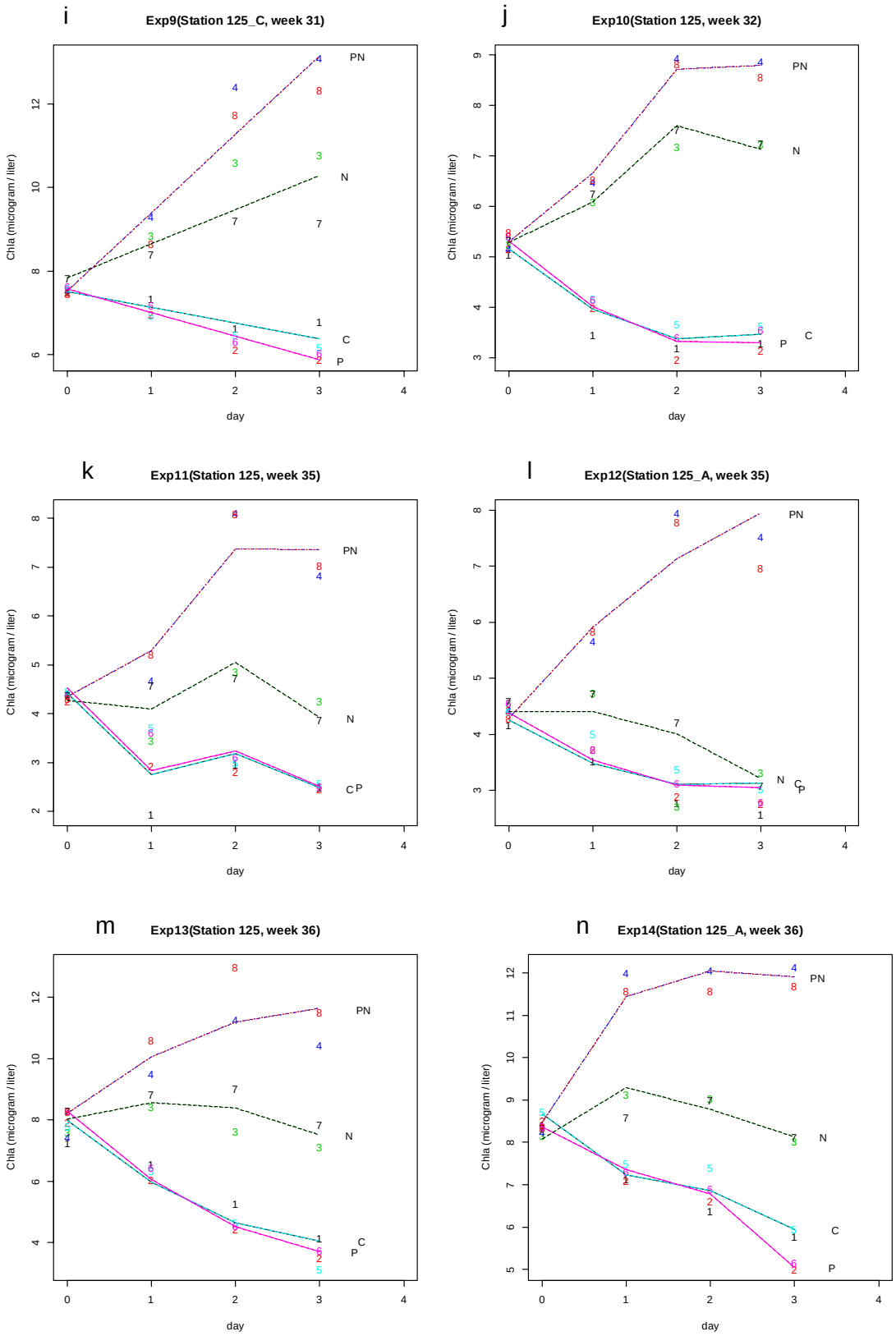
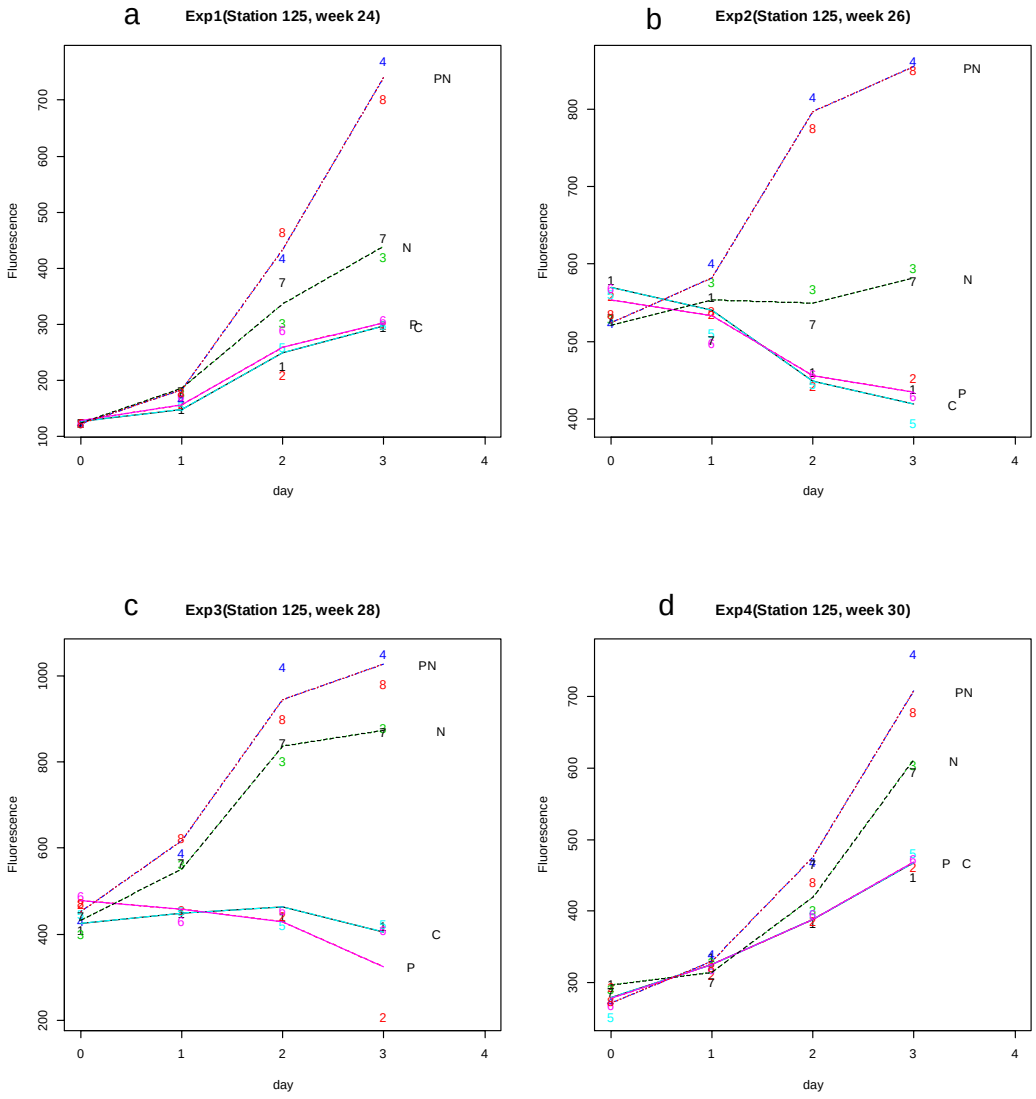


Figure 12. Chlorophyll-a concentration response models of all fourteen experiments.

5.4 Chlorophyll fluorescence measurement results for all the 14 experiments

The final models of all chlorophyll-a fluorescence measurement methods were generated with *p* value method and are shown in Figure 13. The response models of all the experiments have clearly shown nitrogen as the limiting nutrient in the Helsinki coastal region.



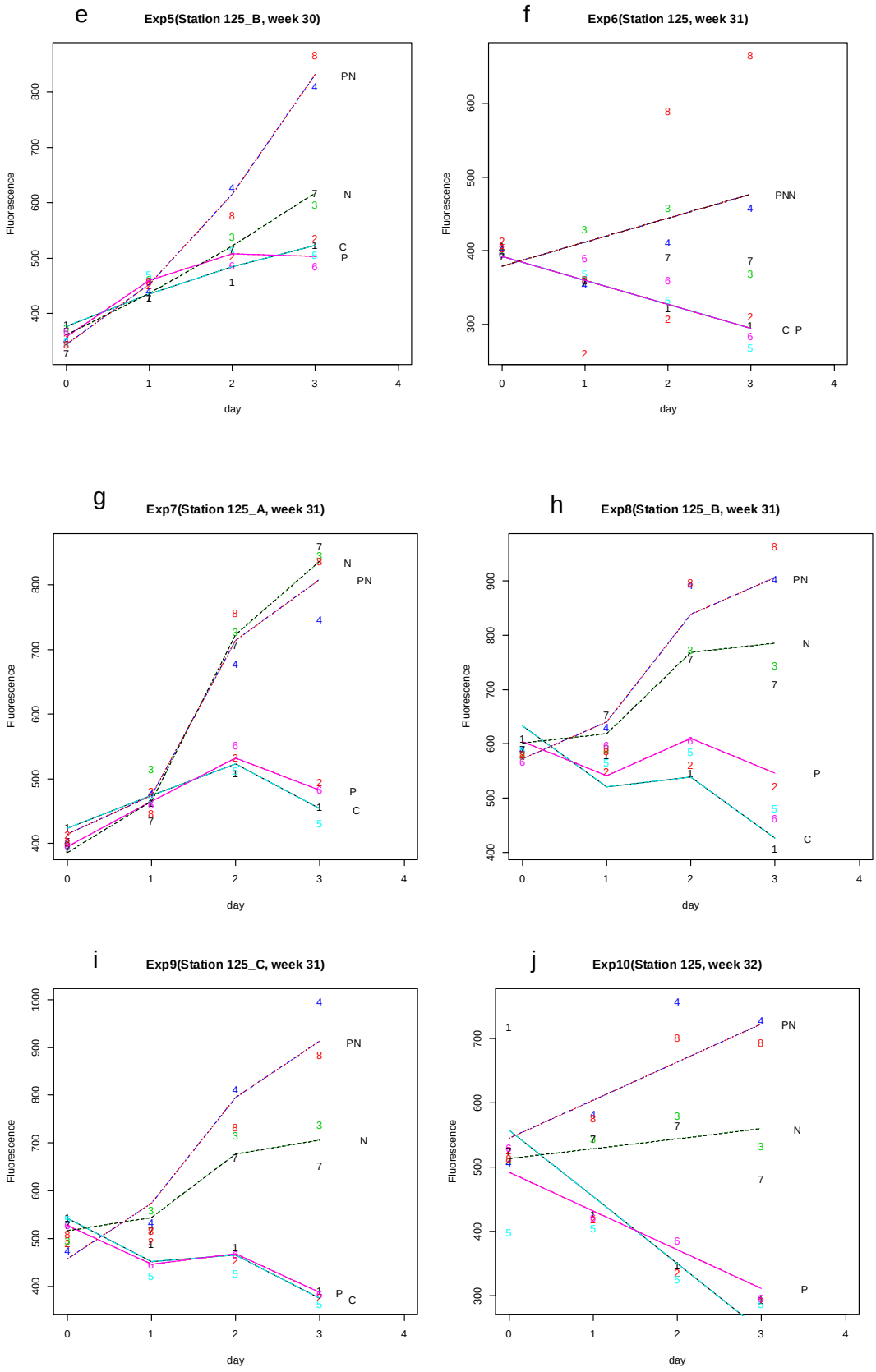


Figure 13. Chlorophyll-a fluorescence response models of all the fourteen experiments.

The above fitted model figures in Figure 12, (chlorophyll-a concentration) and in Figure 13, (chlorophyll fluorescence) show the response behavior of the phytoplankton to the added nutrients. These figures also help us to categorize the different experiments into various classes of nutrient limitation. The summary of these experiments can also be presented in a table by giving colors to the significant coefficients. This can be obtained from the regression summary table of each experiment in R. Significance of coefficients is categorized by the number of stars presented in the regression summary table. The colors are given in such a way that the purple represents 3-star, blue represents 2-star, and light-green represents 1-star and white colors indicate no significance. The level of significance of the stars is described in regression summary table.

The significant coefficients and the model plot provide us information about the phytoplankton behavior on the treatments. A regular monitoring process, for example as explained in this thesis, contains large number of experiments and it has to be reported to the higher authority and for whom reading through all the numerical coefficients and the model plot to find out the phytoplankton behavior on treatment can be a tiresome and time consuming task, also the significant coefficients and the model plot only are not enough to find out the various classes of nutrient limitation. Therefore, a color representation of the significant coefficients and tabulation of the different classes of nutrient limitation is an effective way of presenting the results.

Table 4. Colour representation of the Chlorophyll-a regression coefficients results of the sampling Station 125. Purple represents 3 stars; blue represents 2 stars, light-green represents 1star of the significance level present in p-statistics summary table. White colors indicate non-significant coefficients.

Station 125 (2000 m) Coefficients	Exp_1 Week 24	Exp_2 Week 26	Exp_3 Week 28	Exp_4 Week 30	Exp_6 Week 31	Exp_10 Week 32	Exp_11 Week 35	Exp_13 Week 36
N								
N:t1								
N:t2								
N:t3								
P								
P:t1								
P:t2								
P:t3								
P:N								
P:N:t1								
P:N:t2								
P:N:t3								
t1								
t2								
t3								

Table 5. Colour representation of the Chlorophyl fluorescence regression coefficients results of sampling Station 125. The color representation is same as described above. Fluorescence measurement was not carried out for Experiment 11 and Experiment 13. n.d. = no data.

Station 125 (2000 m) treatments	Exp_1 Week 24	Exp_2 Week 26	Exp_3 Week 28	Exp_4 Week 30	Exp_6 Week 31	Exp_10 Week 32	Exp_11 Week 35	Exp_13 Week 36
N							n.d.	n.d.
N:t1							n.d.	n.d.
N:t2							n.d.	n.d.
N:t3							n.d.	n.d.
P							n.d.	n.d.
P:t1							n.d.	n.d.
P:t2							n.d.	n.d.
P:t3							n.d.	n.d.
P:N							n.d.	n.d.
P:N:t1							n.d.	n.d.
P:N:t2							n.d.	n.d.
P:N:t3							n.d.	n.d.
t1							n.d.	n.d.
t2							n.d.	n.d.
t3							n.d.	n.d.

Table 6. Colour representation of the Chlorophyll-a concentration results of the Sampling Stations125_A, 125_B and 125_C. The color representation is same as described above.

Station 125_A (250 m) treatments	Exp_7 Week 31	Exp_12 Week 35	Exp_14 Week 36	Station 125_B (500 m) treatments	Exp_5 Week 30	Exp_8 Week 31	Station 125_C (1 km) treatments	Exp_9 Week 31
N				N			N	
N:t1				N:t1			N:t1	
N:t2				N:t2			N:t2	
N:t3				N:t3			N:t3	
P				P			P	
P:t1				P:t1			P:t1	
P:t2				P:t2			P:t2	
P:t3				P:t3			P:t3	
P:N				P:N			P:N	
P:N:t1				P:N:t1			P:N:t1	
P:N:t2				P:N:t2			P:N:t2	
P:N:t3				P:N:t3			P:N:t3	
t1				t1			t1	
t2				t2			t2	
t3				t3			t3	

Table 7. Colour representation of the Chlorophyll-a fluorescence results of the Sampling Stations 125_A, 125_B and 125_C. The colour representation is same as described above.

Station 125_A (250 m) treatments	Exp_7 Week 31	Exp_12 Week 35	Exp_14 Week 36	Station 125_B (500 m) treatments	Exp_5 Week 30	Exp_8 Week 31	Station 125_C (1 kmm) treatments	Exp_9 Week 31
N		n.d.	n.d.	N			N	
N:t1		n.d.	n.d.	N:t1			N:t1	
N:t2		n.d.	n.d.	N:t2			N:t2	
N:t3		n.d.	n.d.	N:t3			N:t3	
P		n.d.	n.d.	P			P	
P:t1		n.d.	n.d.	P:t1			P:t1	
P:t2		n.d.	n.d.	P:t2			P:t2	
P:t3		n.d.	n.d.	P:t3			P:t3	
P:N		n.d.	n.d.	P:N			P:N	
P:N:t1		n.d.	n.d.	P:N:t1			P:N:t1	
P:N:t2		n.d.	n.d.	P:N:t2			P:N:t2	
P:N:t3		n.d.	n.d.	P:N:t3			P:N:t3	
t1		n.d.	n.d.	t1			t1	
t2		n.d.	n.d.	t2			t2	
t3		n.d.	n.d.	t3			t3	

The color representation of the chlorophyll-a concentration and chlorophyll fluorescence measurement shows almost same pattern in significant level. These tables help in visualizing the limiting nutrients faster than going through the result output of all experiments and choosing the significant coefficients.

5.5 Classes of limitation

The color representation of the coefficients helps us to distinguish the highly significant, significant and non-significant coefficients. This makes it easier to understand the response models. The main aim of the whole experiments is to find out the limiting nutrient in the coastal region of Helsinki. As discussed earlier in nutrient limitation classes, it is certain that while treating the sea water with two nutrients, nitrogen and phosphorus, the final model obtained will match one of the seven possibilities of nutrient limitation.

To achieve it, the nutrient classes of the each experiment are shown in a table. The selection of the nutrient classes is done by comparing the model of each experiment with the different classes of nutrients explained in background section and Figure 4.

The Table 8 shows that 13 experiments out of 14 show nitrogen limitation in the Helsinki coastal areas, out of which four experiments show exclusively nitrogen limitation and nine experiment show primary nitrogen limitation. However, Experiment 12 is an exception and shows an exclusive PN limitation.

Table 9 shows almost the same result as shown by Table 8. The only difference is in Experiment 8; chlorophyll concentration shows it as primary N limited and chlorophyll fluorescence shows it as primary PN limited. However; this difference is not that big and can be due to noise effect.

Table 8. Nutrient limitation classes of Chlorophyll-a concentration responses

	Exp1	Exp2	Exp3	Exp4	Exp5	Exp6	Exp7	Exp8	Exp9	Exp10	Exp11	Exp12	Exp13	Exp14
Exclusive N (XN)			x	x		x	x							
Primary N (N1)	x	x			x			x	x	x	x		x	x
Exclusive P (XP)														
Primary P (P1)														
Exclusive PN (XC)												x		
Primary PN (C1)														

.

Table 9. Nutrient limitation classes of Chlorophyll Fluorescence response.

	Exp1	Exp2	Exp3	Exp4	Exp5	Exp6	Exp7	Exp8	Exp9	Exp10	Exp11	Exp12	Exp13	Exp14
Exclusive N (XN)						x	x				n.d	n.d	n.d	n.d
Primary N (N1)	x	x	x	x	x				x	x				
Exclusive P (XP)														
Primary P (P1)														
Exclusive PN (XC)														
Primary PN (C1)								x						

The classification of the experiments into various nutrient classes was carried out visually by human eye. The model plot of the first ten experiments of chlorophyll-a concentration and the chlorophyll fluorescence show that the sea water is nitrogen limited. Even though the values and units of the two parameters are different, the fitted lines of the two methods show same response pattern for the same experiment. Some experiments such as the Experiment 3, Experiment 4 and Experiment 8 are nitrogen limited but they fall in different classes of limitation. In chlorophyll concentration the Experinet 3, Experiment 4 and Experiment 8 are exclusively nitrogen limited but in fluorescence method Experiment 3 and Experiment 4 are primary N limited. The differences seen between the various nutrient classes are due to noise effect in fluorescence measurement. If the noise effect is neglected, the fluorescence meaurement show the same nutrient classes as shown by the chlorophyll concentration. However, both of this methods do not show that the water is phosphorus limited.

5.6 FlowCam Test

The taxonomical identification of the particles was done with FlowCam and with a microscope. The FlowCam was used in laboratory to measure the particle density and to identify the particles present in the water. The working principle of FlowCam and the experiment procedure are explained in the methodology section. FlowCam measure-

ments were done on Day 0 for the *control* and on Day 3 for all four treatments (*control, P-alone, N-alone, PN-combined*). Each sample was run for an hour in FlowCam. All samples of Station 125 were run through FlowCam, but the samples from Stations 125_A, 125_B, 125_C were not measured in FlowCam due to lack of time (as on Day 3 it is only possible to measure *control, P-alone, N-alone, PN-combined* treatments of one experiment). The Figure 14 shows the picture of the phytoplankton present in the sample water of Experiment 6, Day 0. Figure 14 shows the presence of filamentous cyanobacteria and diatoms which are prominent in the Gulf of Finland. The identification of all the species measured by FlowCam is not included in this thesis as the taxonomical separation of the particles was not part of this thesis.

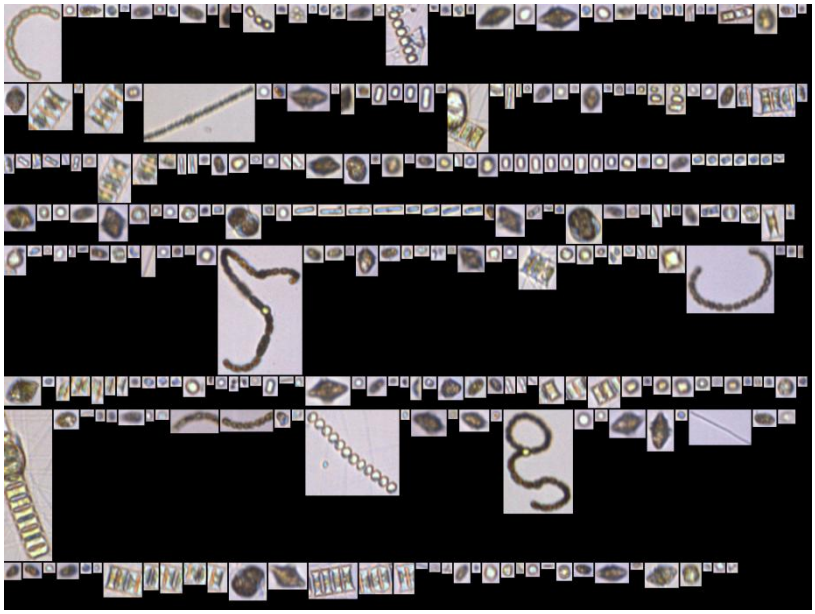


Figure 14. Particles pictures present in sample water of Exp_6, Day 0 taken by FlowCam. No explanation is given in this thesis about the diversity of micro-organisms seen in the picture. The objective was only to find a way of studying the taxonomy but not explaining it.

Each water sample on Day 0 and Day 3 was run in the FlowCam for an hour. Various experiments in FlowCam have shown that it measures 15 ml of water in an hour. FlowCam measurement shows that on Day 3, all four treatments' particle densities are higher than on Day 0. This means the living organisms in the water are responding to the nutrient additions. The Day 0 shows particle density at the time of sampling and Day 3 shows the particle densities due to the treatments. *P-alone* treatment has decreased the biomass whereas; *N-alone* and *PN-combined* treatments have shown more growth than the *control* and *P-alone* treatment.

Table 10. Particle density of the Experiment 6 measured by FlowCam. (Station 125, Week 31).

Treatments	Number of Particles	Particle density (particles / ml)
Day 0	13349	890
Day 3, *control*	14722	982
Day 3, *P-alone*	13983	933
Day 3, *N-alone*	16109	1074
Day 3, *PN*	20810	1388

The Table 10 shows an increase in particle density on Day 3 in *N-alone* and *PN-combined* treatments and no significant increase in Day 3 *control* and *P-alone* treatment. This response shown here is similar to chlorophyll fluorescence and chlorophyll concentration.

5.7 Microscopic test

The taxonomical identification and particle density measurement were carried out as a part of microscopic test. The samples of Day 0 and Day 3 of all experiment were taken for measurement, but in this thesis only the result of Experiment 6 (Station 126, Week 31) and Experiment 7 (Station 125_A, Week 31) are included.

Microscopic test in Experiment 6, as shown in Figure 15a, showed higher biomass at Day 0 and lower on Day 3. *PN-combined* treatment on Day 3 has the lowest biomass as compared to N and P treatment. In Experiment 7, as shown in Figure 15b, biomasses density has increased in *control*, *N-alone* and *P-alone* treatments but has decreased sharply in *PN-combined* treatment. These results did not show the same responses as shown by the chlorophyll-a concentration, chlorophyll fluorescence and FlowCam results. The results obtained by microscopic test did not show any definite behaviour of the phytoplankton.

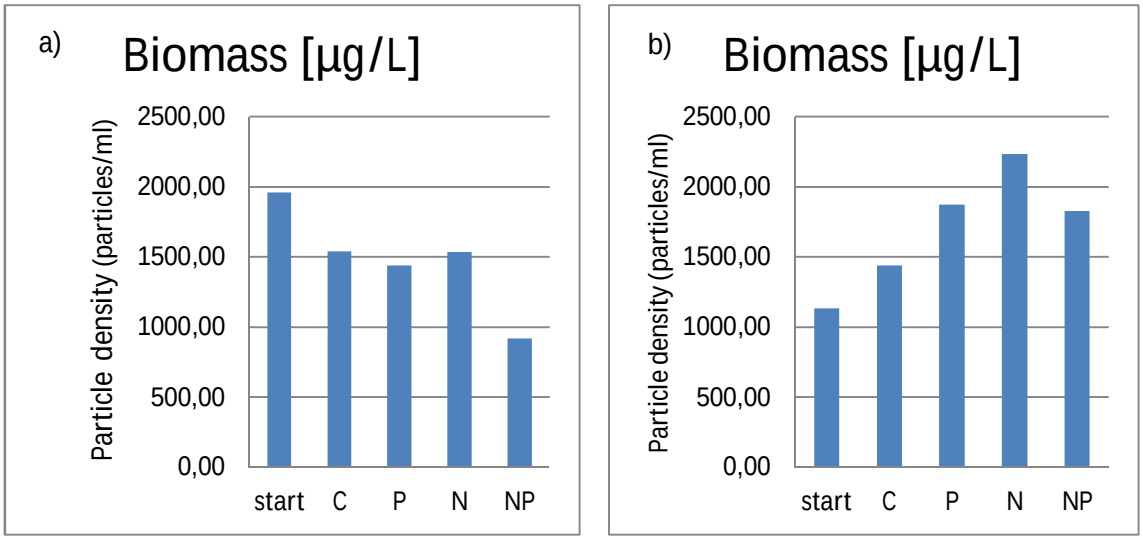
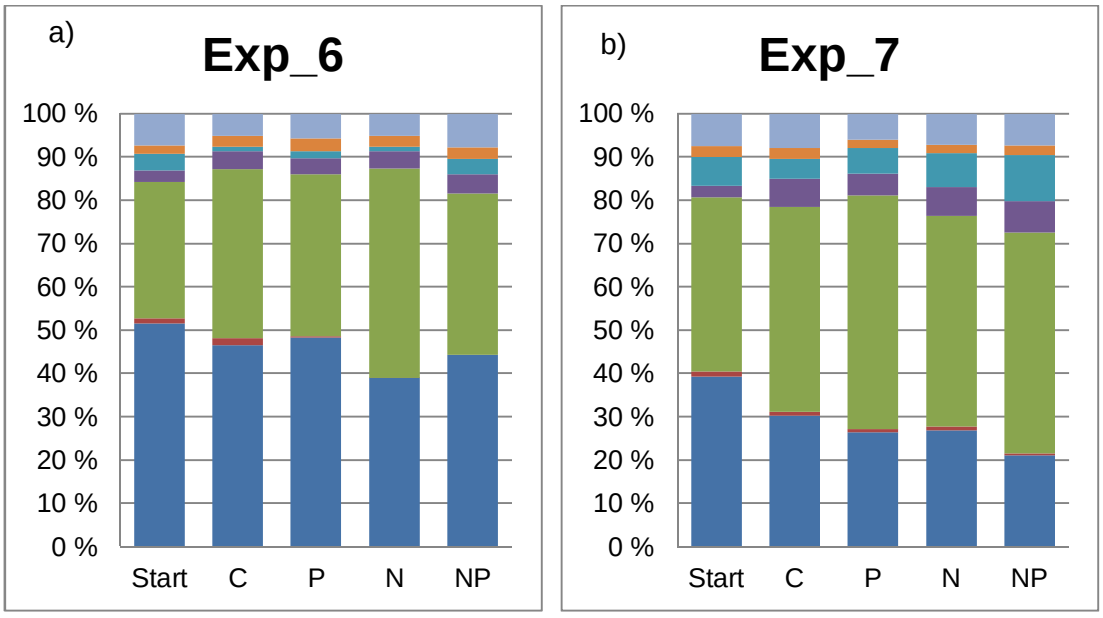


Figure 15. Graphical representation of the total biomass densities [µg/L] obtained from microscopic test. Figure 15a is Experiment 6 results and Figure 15b is Experiment 7 results. The'start' column is the total biomass on Day 0 and Columns 'c*ontrol, P-alone, N-alone, PN-combined'* represent the total biomass of Day 3 of the respective experiments.

The microscopic test of Experiment 6, as shown in Figure 16a, reveals that cyanophyta and dinophyta are the dominant classes of phytoplankton present in the coastal areas. The cryptophyta is present only in the *control* treatment and is absent in others. The community of zooflagellates, chlorophyta and chrysophyta are comparatively smaller than the cyanophyta and dinophyta. Figure 16b shows Experiment 7 microscopic result, which is almost the same as that of Experiment 6. However, Cryptophyta is absent in nutrient treatments in Experiment 6, while it is present all treatments in Experiment 7.



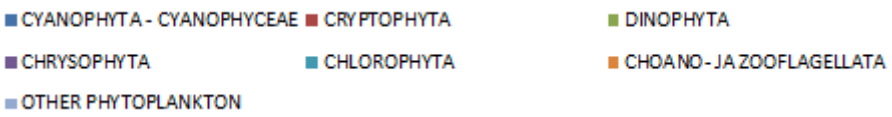


Figure 16. Phytoplankton composition in Experiment 6 and Experiment 7. Figure 16a represents the percentage composition of phytoplankton in samples of Experiment 6 (Station 125, Week 31) and 16b of Experiment 7 (Station 125_A, Week 31).

6 DISCUSSION

6.1 Effluent discharge in Helsinki Sea area and need of regular monitoring

The Helsinki coastal region is regularly loaded with the treated waste water form Viikinmäki waste water treatment plant (for details refer background section) and run-offs from agricultural farms and forestry. The discharged water contains inorganic nitrogen and phosphates that can stimulate the growth of phytoplankton during spring and summer. In order to study the effects on the sea-ecosystem due to nutrients input (explained in the background section) from the effluent of Viikinmäki waste water, a regular monitoring process was carried out by the environmental sector of the City of Helsinki. Any severe signs of eutrophication or any other effects caused by eutrophication can be observed in a regular monitoring process and this can raise an alarm for the responsible bodies to check, and if possible change the treatment system so that the discharged water is not fatal for the aquatic life. This experiment is also a part of the monitoring process.

Phytoplankton need inorganic nitrogen (nitrates or ammonium) and inorganic phosphorus (phosphates) at certain concentration for their growth and these nutrients might not be available to phytoplankton in consumable form or sea water might have only one nutrient and severely lack the other essential nutrient. Under such condition, the concentration of certain nutrients will be high in the water but the signs of severe eutrophication will be absent. However, addition of the limiting nutrient can fulfill the nutrient demand and can lead to algae bloom within short period of time. This shows that measuring the concentration of the nutrients such as different forms of nitrogen and phosphorus present in water is important, but not sufficient to predict the behavior of the phytoplankton responses to required nutrient availability. Thus, further steps (phytoplankton response modeling and taxonomical study) were taken after measuring the nutrient concentration to observe the phytoplankton growth behavior in presence of sufficient nutrients.

6.2 Experimental design, statistical methods and result presentation

A 2^2 factorial experiment design was carried out to study the response of phytoplankton to the addition of limiting or potentially limiting nutrients. In presence of suitable irradiance and temperature, the experiment was carried out with four time points (four days). The factorial design gives interaction effect of nitrogen and phosphorus and this provides the information to study the co-limitation pattern of the nutrients. The co-limitation pattern of the nutrients or the interaction between the added nutrients is very important because addition of only the limited nutrient can again limit the already available nutrient in the water. In the experiments which are classified in the primary nitrogen limited classes (refer Table 8 and Table 9), on addition of only nitrogen in the water has cancelled the nitrogen limitation effect but has made the water phosphorus limited. In such cases, phytoplankton growth stops and supply of more phosphorus is required for more growth. At this point, supply of phosphorus result in higher growth as shown by the *PN-combined* responses. This shows the interaction effect is different than the individual effects and is significant. Another example of the importance of the interaction effect is shown by Experiment 12 (exclusively *PN* limited), here the effects of *P-alone* and *N-alone* treatments behave in the same way as the *control* whereas the combined effect differs from the other treatments. From Experiment 12, if the interaction effect is removed, the model shows that the individual nutrients addition do not give any responses. Thus, a wrong conclusion can be made that on addition of nutrients into the coastal areas will not cause any eutrophication because the model shows same response in absence and presence of single nutrients (only P or only N).

The chlorophyll-a concentrations' model plots of Experiment 1, Experiment 4 and Experiment 5 (refer Figure 12) do not show any difference between the responses till the second day of the experiment. This may be due to the presence of nutrients in the sea water that can be available for phytoplankton for some time. Making any conclusion on the results of the second day would not be enough to define any nutritional classes for the experiments. Major effect of the added nutrients in most of the experiments has been seen only on Day 2 and Day 3 results. Taking four time points for measuring the phytoplankton growth responses was enough to model the eutrophication in the coastal areas. Taking more than four time points was discarded because the volume of water in the 1l bottles was getting lesser everyday due to filtration and the previous similar experiments in marine laboratory has shown that the *in situ* condition of the sea can hardly be obtained in the experimented bottles after 4 days.

The fitted models were plotted on the response chlorophyll-a data and they showed how the phytoplankton would behave in the presence and absence of either or both nutrients. The classification of each experiment result in different nutrient classes was carried out by comparing the fitted model figures (Figure 12 and Figure 13) with the standard nutrient classes' figures (Figure 4), (Andersen and Tamminen, 2007). The nutrient classes show the Helsinki coastal region as nitrogen limited. This conclusion was only possible with the factorial design experiment and this would not have been possible by only measuring the total phosphorus and total nitrogen concentration of the sampled water.

6.3 Chlorophyll responses

Chlorophyll-a concentration and chlorophyll fluorescence were two different parameters measured to classify the sample water of each experiment into different nutrient classes (Beardall et al., 2001). It was easy to compare the results of the two different methods because the numbers of measurements were the same and the same R-script was used for analyzing the data. The results obtained from both methods were similar to each other. The model plots of the significant coefficients of both methods show the growth of the phytoplankton in presence of nutrient addition.

In all the experiments, it can be seen clearly that the nitrogen alone addition has significant positive effects on the phytoplankton growth whereas phosphorus alone addition shows the same response as shown by the *control*. The c*ontrol* has the concentration of nutrients that was present in the sea water at the time of sampling. When phosphorus alone was added to the water, the phytoplankton biomass did not increase significantly. This shows that the phosphorus is already available to the phytoplankton in the sea water and further phosphorus addition does not stimulate phytoplankton growth. Phosphorus addition stimulates the growth only when nitrogen presence is enough in the water. This can be seen in the *PN-combined* responses.

6.3.1 Chlorophyll-a concentration results

For Station 125, which is 2 km away in the direction of discharge effluent, the initial two experiments (Exp_1 & Exp_2, Figure 12a and Figure 12b) were carried out in the second and third week of June 2013, and the last two experiments (Exp_11& Exp_13, Figure 12k and Figure 12m) were carried out at the end of August and beginning of September. All experiments at this sampling point show primary nitrogen limitation of phy-

toplankton. The remaining experiments which were carried out in June and in the beginning of August exhibited exclusive nitrogen limitation. This shows a pattern that phosphorus was used by phytoplankton at the beginning (more sunlight and temperature increasing) and at the end (less sunlight and temperature decreasing) of the summer. However, in Mid-summer (with experiments 3, 4, 6, and 10), the average temperature and irradiance were quite stable, and the added phosphorus was not used by phytoplankton as the responses for nitrogen alone and *PN-combined* show the same result. These shows that in mid-summer, microalgae were not affected on addition of P at all, which means the amount of P in the water was enough for their growth or they have another source of phosphorus which was not present in the beginning and at the end of the summer. However, the effect of temperature and irradiance cannot be speculated on the basis of this data.

Station 125_A is 250 m away in the direction of discharge effluent and sampling was done three times, at the end of July (Exp_7) and August (Exp_12) and in the beginning of September (Exp_13). Experiment 7 and Experiment 13 showed nitrogen limitation but the Experiment 12 was different from the other experiments. In Experiment 12, phytoplankton did not respond to the individual nutrients, but responded only when both the nutrients are available. This shows that during the week, the concentration of the available phosphorus and nitrogen in the water might have been very low for phytoplankton's uses.

Station 125_B is 500 m away in the direction of the discharged effluent and sampling was done twice, on the last two weeks of July (Exp_5 and Exp_8). Results of both experiment showed primary nitrogen limitation. Sample 125_C is 1000 m away in the direction of discharged effluent and sampling was carried out once at the end of July (Exp_9). The result showed primary nitrogen limitation.

In terms of nutrient limitation classes, none of the results of chlorophyll-a concentrations were in no response (00), exclusive phosphorus (EP) and primary phosphorus limitation (P1) classes. All the results showed nitrogen limitation, either (exclusive of primary). Same result was obtained by conducting similar experiment in 1993-1994 by Andersen and Tamminen, 2007.

6.3.2 Chlorophyll Fluorescence results

Chlorophyll Fluorescence measurements were done for the first ten experiments. Various parameters of the photosynthetic processes were measured by the Aquapen fluorometer, but only the minimum fluorescence (F_0) value, fluorescence intensity at 50 µs, was used to plot the model. The treatments having more chlorophyll concentration gave more fluorescence when a light of certain wavelength (450 nm in this case) was passed through it. Out of the ten experiments, the results of three experiments (Exp_4, Exp_5 and Exp_8) slightly differed from the chlorophyll-a concentration results. In the chlorophyll-a concentration measurement, results for Experiment 4 and Experiment 5 exhibited exclusive nitrogen limitation whereas in chlorophyll fluorescence measurement, the results showed primary nitrogen limitation. The differences were very small as could be seen in the model plot and this can be due to some noises in the Aquapen device. The chlorophyll fluorescence of Experiment 8 (Station 125_B, Week 31) was Primary combine limited (*PN1*), which shows that even phosphorus alone addition receives more response than the *control;* whereas the chlorophyll concentration for the same experiment indicated primary nitrogen limitation (N1), which shows that phosphorus alone addition was similar to the *control* and did not have any significant effect. The chlorophyll fluorescence measurement of Experiment 5 (Station 125_B, Week 30) whose sample came from the same sampling station and had been sampled just a week before has showed exclusively nitrogen limitation. Considering fluorescence result of Experiment 8, it can be concluded that the small response shown by fluorescence in phosphorus alone addition was not significant and was due to some noise effects in the measurement. Chlorophyll fluorescence results, like chlorophyll-a concentration results, suggest that the water in the Helsinki coastal region is nitrogen limited.

6.3.3 Cost comparison of the chlorophyll responses methods

The results of chlorophyll fluorescence's were similar to the results of chlorophyll-a concentrations. Observing and studying the classes of nutrient limitation is possible by using either of the methods. The cost of the two methods is described below.

In chlorophyll-a concentration measurement, the cost of one GF/F glass filter is around 60 Euro cents and more than 64 glass filters (some filters get destroyed during filtration) are needed for one experiment as designed and explained in this thesis. Around 700 ml of ethanol (96% by weight) is needed for extracting chlorophyll- a (10 ml per

sample) which cost approximately 10 €. An experienced laboratory technician takes around 45 minutes for filtering sixteen samples every day and around 90 minutes to measure the 64 samples. Thus, the total filtration and analysis takes around 4.5 to 5 hours for one experiment setup. The wage of an experienced laboratory technician is around 3000 € per month (including social cost) for 144 hours. Hence, the wage of a laboratory technician for conducting one experiment is around 110 € and the total cost of conducting chlorophyll-a concentration measurement for one experiment is around 160 €.

In Fluorescence concentration measurement, excluding the price of cuvettes and Aquapen (these are provided by laboratory), the only cost is the wage of the laboratory technician. Analysis of one day result takes around half an hour (maximum estimation) thus for one experiment set up, the time needed is 2 hours. Assuming the same wage of laboratory technician as mentioned earlier, the wage of two hours is around 42 €.

The cost estimation shows that the fluorescence method is four times cheaper and much faster than the chlorophyll-a measurement. If the cost of laboratory technician is excluded, the final cost of chlorophyll-a concentration would be around 50 € and chlorophyll fluorescence have no cost at all. The difference in cost of the two methods for conducting one experiment is not so huge for an environmental body or for a marine laboratory, but for a continuous monitoring process, when hundreds experiments are conducted, the operational cost differences of the two methods plays a big role in determining the budget. For a regular monitoring process, fluorescence result would be the best choice in terms of cost and time. In addition, chlorophyll fluorescence is also important for phycological study as it measures the photosynthetic activity of the cell and the physiology of the cell before and after nutrient treatments can be studied. Cost estimation values are provided by Marine research laboratory (SYKE).

6.4 Phytoplankton taxonomy and density

The taxonomical identification, particle counting and density measurement of the phytoplankton were done by FlowCam method and microscopic test method. In this thesis, FlowCam measurement (only Experiment 6) and microscopic test (only Experiment 6 and Experiment 7) are discussed.

6.4.1 FlowCam results

FlowCam measures the particle number and its particle density can be calculated when the time period of FlowCam sampling is known. One of the main advantages of flow cam over microscope is that there is no need of a specialist while running the sample and the files can be saved for future purposes. Identification of the organisms in FlowCam can be done by a microbiologist in computer, at any time after storing the results, which is much easier, reliable, cheaper and faster than traditional microscopic test method. Figure 14 shows the pictures of the phytoplankton present in c*ontrol* of Day 0 of Experiment 6. The FlowCam result of the Experiment 6 revealed that the *N-alone* and the *PN-combined* treatments had increased the number of phytoplankton whereas the *control* and the *P-alone* treatment had almost the same particle number. This result shows the same response behavior of phytoplankton towards the nutrient treatment as shown by the chlorophyll-a concentration and chlorophyll fluorescence results. FlowCam measures the entire particle that passes through it in given time and thus the data obtained from its reading is statistically significant. For a more precise result, particle number can be counted by flow cytometer (data not included in this thesis).

6.4.2 Microscopic results

Microscopic test is a traditional and time consuming method of taxonomical classification of microorganism. This test is usually carried out with very small fraction of phytoplankton community, with a sub selection part of the filtered biomass (in Experiment 6 and Experiment 7, only 2000 particles from each treatment was counted), thus the data obtained from the test might not include all information actually revealed by the phytoplankton responses. The microscopic results of Experiment 6 show decrease in total biomass in nutrient addition whereas Experiment 7 result is also not promising as the *PN-combined* treatment has the lowest biomass. In microscopic test only few clusters of microorganisms are counted out of many clusters and these excluded clusters could contain the main microorganisms affected by the nutrient treatments; therefore chances of losing reliable microscopic information is higher than with the other methods.

6.4.3 Phytoplankton test reliability and cost comparison

The two tests on taxonomical identification and particle density measurement of phytoplankton suggest that with FlowCam it is much faster and easier to find out the domi-

nant phytoplankton in the samples. Experiment 6 has five phytoplankton samples and it takes days for a microbiologist to complete the taxonomical and particle density measurement, whereas FlowCam takes few hours for a trained microbiologist to identify the different species in a computer. FlowCam itself calculates the particles density present in the sample. In terms of cost, for regular monitoring processes, FlowCam provides results much faster; thus it is much cheaper than microscopic test.

6.5 Nutrient Limitation in the sampling site

A bioassay experiment to reveal the nutrient limitation pattern conducted in 1993-1994 has shown nitrogen as the limiting nutrient in Helsinki coastal areas (Andersen and Tamminen, 2007). The nutrient limitation classification in their experiment has revealed primary N limitation as the dominant nutrient limitation class, followed by exclusive N limitation and exclusive combine limitation on the Day 2 and Day 3 chlorophyll-results. The Portion of phosphorus limitation was very small on Day 2 and absent in Day 3, which concludes that the nitrogen plays dominant role in stimulating the phytoplankton growth and phosphorus is effectively used by phytoplankton only when the supply of nitrogen is sufficient (Andersen and Tamminen, 2007). A similar bioassay experiments was conducted during this thesis project in the Helsinki coastal area in the summer of 2013 and the results also showed nitrogen as the limited nutrient. In this thesis, classification into various nutrient limitation classes was done by comparing the model plots with the figures of limitation classes presented by Andersen and Tamminen (2007) in their study.

Both Andersen and Tamminen (2007) and this thesis project showed nitrogen as the limiting nutrient in Helsinki coastal region and maximum algae bloom with the combined nutrients addition. The results of this thesis project show some shifts in the limitation classes especially at Station 125, which was regularly monitored throughout the summer of 2013. At this station the nutrient limitation class is primary nitrogen limited (P1) at the beginning and at the end of the summer but is exclusively nitrogen limited (XN) in mid-summer. To receive more accurate results in limitation classes, the statistical software used previously by Andersen and Tamminen should be updated and make it computable for modern computers (unfortunately nutrient limitation statistical software by Andersen and Tamminen did not work with modern computers). Further studies on the phytoplankton taxonomy using FlowCam, and on the changes in the photosynthetic activities using chlorophyll fluorescence measurements, as well as use of

modern statistical software in analyzing the data would give more detailed results on phytoplankton behaviors that change with time, nutrient and environmental conditions.

7 Conclusion

The factorial design experiment set up with time series provided *in situ* conditions for the phytoplankton growth even though the experiment setup lacked some of the natural phenomena, for example, the shifts in temperature during day and night, continuous movement of water, change in irradiance during the week, concentration of supplied nutrients and continuous grazing effects. However, the main objective was to study the effects of added nutrients in the phytoplankton community assuming that the phytoplankton responses to additional nutrients in the sea with changing environmental conditions would not vary significantly from the responses that were simulated in the laboratory conditions. The results show excess addition of nitrogen in sea water through any sources will accelerate the growth and such nitrogen sources should be monitored regularly to minimize nitrogen loading into the Baltic Sea.

Results obtained from various methods carried out in the laboratory suggest that phosphorus is sufficiently available to the phytoplankton and nitrogen is the main nutrient that is limited and its availability can trigger eutrophication rate. This result shows no change in limiting nutrient in 20 years and is consistent with that of a similar bioassay experiment by Andersen and Tamminen conducted in 1993-1994. As a main point source, Viikinmäki wastewater treatment plant should continuously monitor their treatment procedures and the nature of effluent discharged into the sea. *P-alone* treatment has shown no significant results but it does not mean that waste water effluent should be monitored only for nitrogen emission. *PN-combined* addition has shown significant results, so even phosphorus emission should be strictly monitored along with nitrogen emission because nitrogen loading into the sea is also possible from other sources such as atmospheric deposition, inputs from river, agricultural runoffs and atmospheric deposition (HELCOM 2009).

Classification of the each experiment into various nutrient classes is obtained by comparing the model plots with the figures of nutrient classes found in Andersen and Tamminen (2007). Model plot of each experiment is the function of the significant coefficients. The significant coefficients not only reveal the main effects and interaction effects of the added nutrients but also show how phytoplankton behaves with respect to

the various time effects (linear, quadratic or cubic). Correct statistical analysis of obtained chlorophyll-a and fluorescence data is important for obtaining the significant coefficients. Normal score plots of the coefficients is a traditional method of choosing the significant coefficients, whereas comparing the *p* value with standard significant level is a statistically reliable and unbiased method for choosing the significant coefficients, which can be easily obtained from the R-software regression summary table.

Chlorophyll-a concentration measurement gives precise results about the limited nutrient and its effects. Similar results were possible with chlorophyll fluorescence measurements in lesser time and with comparatively lower cost. Fluorescence dataset is huge and it includes various variables describing state of cell physiology and it is also possible for an expert to study the changes in photosynthetic process in algae cells before and after the nutrient treatments.

Taxonomical identification of phytoplankton and particle density measurement with microscope was found very costly, time consuming and the obtained microscopic result did not match with the other results. Even though the taxonomical identification of the phytoplankton was successful with microscope, the biomass results obtained was totally different from chlorophyll-a concentration, fluorescence and FlowCam results. Taxonomical identification of the particles is also possible with FlowCam and its biomass results showed similar behavior of phytoplankton to that shown by the chlorophyll-a concentrations and fluorescence measurements.

Regular monitoring is necessary to study the changes caused by the discharged waste water in the coastal areas. Considering huge cost requirements for regular monitoring process, this thesis has given some suggestion in getting reliable results with minimum expenses. The new methods were found much cheaper and faster with equally reliable results than the traditional methods. In future, more experiments can be successfully conducted with limited budget if fluorescence measurements and FlowCam methods are used instead of measuring chlorophyll-a concentration and conducting microscopic tests.

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Appendix 1

A. Materials

Following materials were used in the experiment for growing the phytoplankton in the sample bottles and measuring the concentration of chlorophyll-a.

Chlorophyll-a measurement

1. Thermostat Water bath
2. Fluorescent tubes (OSRAM L 58W/965)
3. Nalgene 1L and 2L Bottles
4. Glass vials (10 ml)
5. Whatman GF/F Glass-microfiber filter, diameter = 25 mm, nominal pore size = 0.7 µm
6. Ethanol (96.1 % by volume and 94 % by weight)
7. Dispensette Organic bottletop dispenser (100 ml)
8. Filtration unit with funnels
9. Filtration pump (vacuum below 200 mbar)
10. Spectrofluorometer (JASCO FP-750)
11. Varian Fluorescence spectrophotometer
12. Pipette (350 µl)
13. Measuring flask (1 L)
14. Eclipse Fluorescence spectrophotometer
15. 96 well plates
16. Milli-Q water (18.2 MΩ.cm)
17. Funnel
18. A bucket (15 l)
19. Nitrogen source (NH_4Cl, Ammonium chloride)
20. Phosphorus source (KH_2PO_4 , Mono potassium Phosphate),
21. Plastic container (25 L)

Chlorophyll fluorescence measurement

22. Aquapen-C-AP-C100 (Photo system instruments, spol.s.r.o)
23. Quartz 10 mm cuvettes

Phytoplankton taxonomic identification and biomass measurement

24. Flowcam (Fluid imaging Technologies Inc. USA)
25. Beaker (Borosilicate, 50ml)
26. Magnetic stirrer

Phytoplankton taxonomic identification and size measurement with microscope

27. Lugol solution
28. dark brown glass bottles (100 ml)
29. Microscope Leica DMI3000 B with accessories

B. Data Processing in R for regression

The treatments, time, replicate bottles, replicate measurements and the response data as arranged in columns in a text file and save it under data1.txt. The R-script for generating the model is in Appendix 1F.

At first the whole text file is read in R.
> data1 <- read.table ('data1.txt',header=TRUE).This command reads the data set.
After sourcing the data in R, the dimension of the data is checked.

```
> dim(data1)
[1] 64 19
```

The data has 64 rows and 19 columns in the data1.txt. There are 8 bottles, 2 replicates from each bottle and 4 measurement days for an experiment. So, 8*2*4=64 responses are obtained for an experiment and each phytoplankton response is represented as row in the data set.

```
> head(data1)
   P  N time R Sample Exp_1 Exp_2 Exp_3 Exp_4 Exp_5 Exp_6 Exp_7 Exp_8 Exp_9 Exp_10 Exp_11 Exp_12 Exp_13 Exp_14
1 -1 -1    0 1      1  3.11  3.58  5.72  5.84  4.50  5.50  4.98  7.70  7.28   5.12   4.55  4.120   8.02   8.68
2  1 -1    0 1      1  3.25  3.82  6.04  5.52  4.38  5.68  5.48  7.76  7.78   5.02   4.14  4.460   7.50   8.00
3 -1  1    0 1      1  3.09  3.76  5.48  5.56  4.52  5.74  5.82  7.66  8.02   5.22   4.20  4.500   6.74   8.68
4  1  1    0 1      1  3.51  3.86  5.76  5.86  4.32  5.38  5.58  7.26  7.98   4.98   4.28  4.635   7.58   7.38
5 -1 -1    0 1      2  3.18  4.04  5.56  6.00  4.30  5.48  6.00  6.92  7.52   5.28   4.36  4.280   7.22   8.12
6  1 -1    0 1      2  2.80  3.82  5.82  6.22  4.10  5.76  5.74  6.96  7.96   5.48   4.24  4.700   8.00   8.00
```

Figure 17. First five rows of the data arranged in text file.

The command head (data1) shows the 19 columns and the first six rows of data1. Column 1 with heading P is the phosphorus treatment, column 2 is nitrogen treatment, column 3 is the time (Day 0 to Day 3), column 4 is the replicate bottle, and column 5 is the replicated measurement. Columns 6 to column 19 represent the 14 experiments.

Now, the average of the replicated measurements of each experiment is calculated and the dimension of the new data set is checked.

```
> data2 <- aggregate(.~P+N+Sample+time,data1,FUN=mean).
```

This makes data2 as a new dataset by taking the average of the replicated measurements.

```
> dim(data2)
[1] 32 19
```

The data2 has new dimension with 32 rows and 19 columns. Initially in data1 has 64 rows and after taking the average of the replicated measurement, the number of rows decreases to 32.

(One response has one replicate in each measurement). The 19 columns are the same as in data1.

```
> T
     [,1] [,2] [,3] [,4]
[1,]    1   -3    1   -1
[2,]    1   -1   -1    3
[3,]    1    1   -1   -3
[4,]    1    3    1    1
```

Figure 18.Orthogonal polynomial matrix.

The columns of the orthogonal matrix T represent various time effects. The first column represents constant effect, which means that there is no growth in biomass even with the additional nutrients. The second column represents the linear time effect, which shows that the phytoplankton growth is linear with time. The third column represents the quadratic time effect and the fourth column represents the cubic time effect, which shows quadratic response and cubic response to the additional nutrients respectively.

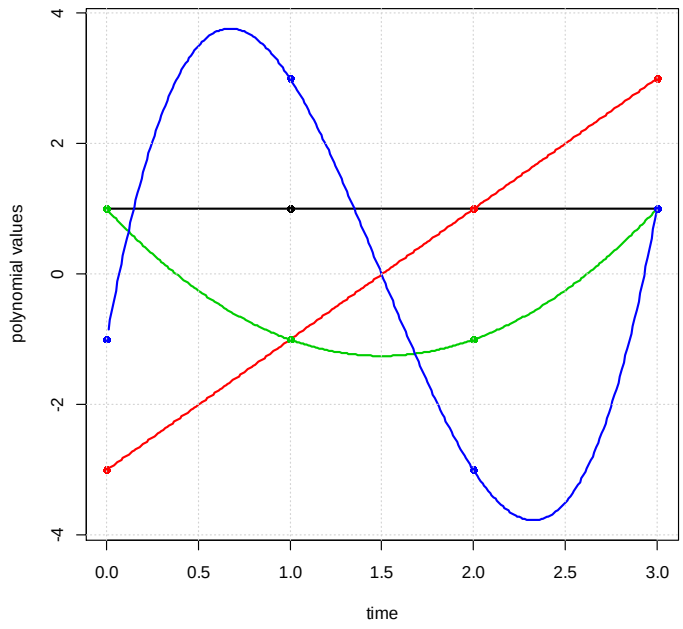


Figure 19. Plot of the orthogonal matrix T. The dots on the lines are the matrix points. The black line is the Zeroth order polynomial, red line is the first order polynomial, green line is the second order polynomial and blue line is the third order polynomial.

The orthogonal matrix T has only 4 rows. Since data2 has 32 rows, 32 rows in the time effects (linear, quadratic and cubic) are required. It can be done by generating equations for linear, quadratic and cubic time effects. The equations for the time effects in R are obtained by using lm function.

```
> tt=0:3
> tt
[1] 0 1 2 3
> lm(T[,2]~tt)

Call:
lm(formula = T[, 2] ~ tt)

Coefficients:
(Intercept)           tt
         -3            2

> lm(T[,3]~tt+I(tt^2))

Call:
lm(formula = T[, 3] ~ tt + I(tt^2))

Coefficients:
(Intercept)           tt      I(tt^2)
          1           -3            1

> lm(T[,4]~tt+I(tt^2)+I(tt^3))

Call:
lm(formula = T[, 4] ~ tt + I(tt^2) + I(tt^3))

Coefficients:
(Intercept)           tt      I(tt^2)      I(tt^3)
     -1.000       15.667      -15.000        3.333
```

Figure 20. Process for obtaining the equations for various time effects.

'tt' represents the measurement days. (Day 0, Day 1, Day 2 and Day 3). From above R commands, the various time effects equations are obtained

Linear time equation (t1) = - 3 + 2 * t

Quadratic time equation (t2) = 1 – 3 * t + t ^ 2

Cubic time equation (t3) = - 1 + 47 / 3 * t – 15 * t ^ 2 + 10 / 3 * t ^ 3

(In our R script, 't' is used instead of 'tt' because 't' is time column in our data.txt)

Model for each experiment is generated using the function *lm* in equation:

y~P*N + (ti+t2+t3)*(P*N) in R software and it is written as

> model1 <- lm(y~(P*N)+(ti+t2+t3)*(P*N))

Where, y is the phytoplankton response to the treatments, P = phosphorus treatment; N= nitrogen treatment; t1 = linear time effect; t2= quadratic time effect and t3= cubic time effect.

'~' is a model operator. It separates the response on its left with the other variables on its right.

The regression Coefficients are shown below.

1. *lm* in R itself generates intercept for the model. Intercept is the mean of all the responses and is the first coefficient of the model.

The model term P*N gives three coefficients. They are

2. main effect of P as **P**
3. main effect of N as **N**
4. Interaction effect of P and N as **P: N**.

The model term (ti+t2+t3)*(P*N) gives 12 coefficients. They are

5. linear time effect as **t1**
6. linear time effect with P as **P : t1**
7. linear time effect with N as **N : t1**
8. linear time effect with P and N interaction as **P : N : t1**
9. Quadratic time effect as **t2**
10. Quadratic time effect with P as **P : t2**
11. Quadratic time effect with N as **N : t2**
12. Quadratic time effect with P and N interaction as **P : N : t2**
13. Cubic time effect as **t3**
14. Cubic time effect with P as **P : t3**
15. Cubic time effect with N as **N : t3**
16. Cubic time effect with P and N interaction as **P : N : t3**

C. Lack-of-fit *F* test

The null hypothesis of lack of fit *F* test states that *there is no lack of fit* and the alternative hypothesis states that there is *lack of fit* in our model (Montgomery. 2001). The average of replicated measurement is taken before making model of the chlorophyll-a data. To conduct the *F* test, the standard deviation of the replicated sample bottles is calculated.

y1 = y[(1:32)[S==1]]

y1 gives the chlorophyll-a data of the first set of bottles.(Bottles 1C,2P,3N,4PN)

y2 = y[(1:32)[S==2]]

y2 gives the chlorophyll-a data of the second set of bottles.(Bottles 5C,6P,7N,8PN)

> (MSpe) <- mean(apply(rbind(y1,y2),2,sd)^2) Mean Square of Pure error

In above code, rbind binds the y1 and y2 in rows such that 1C and 5C, 2P and6P, 3N and 7N, 4 PN and 8PN of all four experimented days are arranged in columns. '*apply'* applies function over array margin. 1 indicated rows and 2 indicate columns.

(http://127.0.0.1:30839/library/base/html/apply.html)

Here it applies standard deviation function over the column of the row-*binded y1 and y2.*

> dfpe <- 16 (degree of freedom of pure error in model1 and is obtained from the model1 summary table)

> SSpe <- MSpe * dfpe (Sum of squares of pure error)

> SSres <- sum(residuals(model2)^2) (Sum of squares of residuals and is obtained from model2 summary table)

> dfres <- model2$df.residual (degree of freedom of residuals and is obtained from the model2 summary table)

> MSres <- SSres / dfres (Mean square of residuals)

> SSlof <- SSres – SSpe (Sum of squares of lack of fit)

> dflof <- dfres – dfpe (degree of freedom of lack of fit)

> MSlof <- SSlof / dflof (Mean Square of lack of fit)

> F <- MSlof / MSpe (F value) (Montgomery. 2001)

Now In R, the *p* value for the *F* test is obtained with the following command

> print(1-pf(F,dflof,dfpe)) (*p* value, significance level = 0.05)

```
> rbind(y1,y2)
    [,1]  [,2] [,3]  [,4] [,5] [,6] [,7] [,8] [,9] [,10] [,11] [,12] [,13] [,14] [,15] [,16]
y1 3.075 3.175 3.23 3.235 4.26 4.07 4.56 4.92 3.54   3.3  4.50  5.98  3.16  3.19  4.40  9.34
y2 3.275 3.110 3.30 3.255 4.14 4.00 4.51 4.37 3.40   3.2  4.47  5.61  3.16  2.87  4.51  8.98
> MSpe
[1] 0.02454141
> SSpe
[1] 0.3926625
> SSres
[1] 0.4655505
> dfres
[1] 19
> MSres
[1] 0.02450266
> SSlof
[1] 0.07288797
> dflof
[1] 3
> MSlof
[1] 0.02429599
> F
[1] 0.9899999
> pf(F,dflof,dfpe)
[1] 0.5775035
> 1-pf(F,dflof,dfpe)
[1] 0.4224965
```

Figure 21. Lack of fit *F* test result for Experiment 1, Station 125, Week 24 (2013).

The R- command *rbind(y1,y2)* shows the chlorophyll-a response data of the replicated bottle samples in the Figure 21 above. The *p* value of the lack of fit *F* test is 0.4224965, which is greater than the standard significance level (0.05), (0.4224965 > 0.05). Since, *p* value is greater than 0.05, the null hypothesis is not rejected and it is ensured that there is no significant lack of fit in the selected model of the Experiment 1.

D. **Least squares method**

x_1 gives the coded value for 2^2 factorial design, where the first column is the mean of all responses, second column is the P addition, third column is the N addition and fourth column is the combined P and N addition.

```
x_1
     [,1] [,2] [,3] [,4]
[1,]    1   -1   -1    1
[2,]    1    1   -1   -1
[3,]    1   -1    1   -1
[4,]    1    1    1    1
```

xt <- kronecker(t,x).

xt is a new matrix formed by kroneker product of t and x. Kronecker product: if t is n*n matrixes and x is m*m matrixes, t⊗x is an nm*nm matrix with block elements. Here, t is 4*4 matrices and x is 8*4 matrixes, so xt would be 32*16 matrixes. Then the coefficients are obtained by following command in R and normal plots are plotted for choosing the significant coefficients.

> b=solve(t(xt)%*%xt)%*%t(xt)%*%(data.matrix(y)) # (Box and Draper, 2007. p.235)
> bvs=solve(sqrt(t(xt)%*%xt))%*%t(xt)%*%(data.matrix(y)) # (Box and Draper,2007. p.240)
> bn=(bn-mean(bn)) # mean centered
> normplot <- qqnorm(bn) # (Box and Draper,2007. p. 241)

E. R script for least squares estimation method

```
graphics.off()

data1 <- read.table('data1.txt',header= TRUE)      # reads the data set
data2 <- aggregate(.~P+N+Sample+time,data1,FUN=mean)
                                        # gives the mean of replicated measurement

y  <- data2$Exp_1                      # experiment number
x_1 = matrix(c(1,-1,-1,1,1,1,-1,-1,1,-1,1,-1,1,1,1,1),ncol=4,nrow=4,byrow=TRUE)
                                        # x_1 gives the coded value for 2^2 factorial design
x=rbind(x_1,x_1)                        # binds x_1 and x_1 in row

t = matrix(c(1,-3,1,-1,1,-1,-1,3,1,1,-1,-3,1,3,1,1),ncol=4,nrow=4,byrow=TRUE)
                                                # orthogonal matrix

xt <- kronecker(t,x)                            # kroneckor product of t and x

b=solve(t(xt)%*%xt)%*%t(xt)%*%(data.matrix(y))    # gives the sixteen coefficients

bvs=solve(sqrt(t(xt)%*%xt))%*%t(xt)%*%(data.matrix(y)) # scales the coefficients

bn=bvs[2:16,]                   # chooses the scaled coefficients except the intercept
bn=(bn-mean(bn))           # mean center the chosen coefficients

normplot <- qqnorm(bn)                          # gives the normplot
plot(normplot$y,normplot$x,xlab='normal standard mean',ylab='normal score',main='Exp_1,
station 125, week 24')              # gives names of x-axis, y-axis and title.

qqline(bn,datax=TRUE)                           # fits the line on normplot

bnp <- b[2:16];                # chooses the scaled coefficients except  intercept

f=locator(n=2,type='n')               # locates point on the graph

a   <- sort(f$x)        #Afterlocating pointsin normplot,sort command sorts the values

of x-axis

bnp[bn>a[1] & bn < a[2]]=0
```

```
                          # The loop makes the coefficients zero which are more than
a[1]  the and less than a[2].

varnames <- c('P',  'N',  'PN',
              'Ct1','Pt1','Nt1','PNt1',
              'Ct2','Pt2','Nt2','PNt2',
              'Ct3','Pt3','Nt3','PNt3')   # names of the coefficients
text(normplot$y[abs(bnp)>0],
     normplot$x[abs(bnp)>0],varnames[abs(bnp)>0])
                                      # gives text of the significant coefficients

bnp <- c(b[1],bnp)                    # puts intercept in the significant coefficients

est=xt%*%bnp                          # gives estimates of the model.

dim(est)=c(8,4)                      # gives the dimension of estimates as 8 rows and 4
col

dim(bnp)=c(4,4) )                   # gives the dimension of bnp as 4 rows and 4 col


r2=sum((est-mean(y))^2)/sum((y-mean(y))^2)
                            # gives the coefficient of determination (R squared value)

yy <- matrix(y,8,4)               # convert y column matrix into 8*4 matrixes

windows()                         # opens new R window

day <- 0:3                        # day= 0 1 2 3

matplot(day,t(yy),type='p',xlim=c(0,4),ylab='Chla   (microgram   /   liter)',main='Exp_1,
station 125, week 24')            # plots the day and yy matrix

matlines(day,t(est))             # fits the model with the estimates

text(jitter(rep(3,4),3)+.2,est[1:4,4],c('C','P','N','PN'))
                               # jitter separates the overlapped treatment names;
                               # text gives the names of the estimates.
```

F. R script for *p* value method(for Exp_1 as example)

```
graphics.off()           # clear the graphic windows
data1 <- read.table('data1.txt',header=TRUE)     # reads the data set
data2 <- aggregate(.~P+N+Sample+time,data1,FUN=mean)
                                      # gives the mean of replicated measurement.
P  <- data2$P                         # P column from data2
N  <- data2$N                         # N column from data2
y  <- data2$Exp_1                     # experiment number
S  <- data2$Sample                    # Sample Bottle replicate
t  <- data2$time                      #  time column (days)
T  <- matrix(c(1,-3,1,-1,1,-1,-1,3,1,1,-1,-3,1,3,1,1),ncol<-4,nrow<-4,byrow<-TRUE)
                                      # orthogonal matrix
t1 <- -3+2*t                          # Linear time equation
t2 <- 1-3*t+t^2                       # Quadratic time equation
t3 <- -1+47/3*t-15*t^2+10/3*t^3       # Cubic time equation
```

```
model1 <- lm(y ~ P*N+(t1+t2+t3)*(P*N)) # gives the main effects and interaction effects.
print(summary(model1))                    # Prints the summary of the model1

model2 <- lm(y ~ P*N+(t1+t2+t3)*(P*N)-P:t3-N:t3-P:N:t3)
                           # removes the non-significant coefficients and makes model2
print(summary(model2))       # Prints summary of model2

#Lack of fit test
y1   <- y[(1:32)[S==1]]                 # y1 refers to the replicate sample(bottle)1
y2   <- y[(1:32)[S==2]]                 # y2 refers to the replicate sample(bottle)2

MSpe <- mean(apply(rbind(y1,y2),2,sd)^2)   # gives the mean square of pure error

dfpe <- 16;                   # degree of freedom of pure error,(obtained from model1).

SSpe <- MSpe*dfpe                      # Sum of squares of pure error

SSres <- sum(residuals(model2)^2)      # Sum of squares of residuals

dfres <- model2$df.residual            # degree of freedom of model2

MSres <- SSres/dfres                   # Mean square of residuals

SSlof <- SSres-SSpe                    # Sum of squares of lack of fit

dflof <- dfres-dfpe                    # degree of freedom of lack of fit

MSlof <- SSlof/dflof                   # Mean square of lack of fit
F     <- MSlof/MSpe                    # gives lack of fit test
print(1-pf(F,dflof,dfpe))              #  gives the p value

qq.plot  <-  qqnorm(residuals(model2),main='Exp_1  residual  plot',ylab='residuals',xlab=
'standard normal distribution')
                           # gives the normal probability plot of residuals of model2

qqline(residuals(model2))              # fits the line in the qq.plot

text(qq.plot$x,qq.plot$y,1:32)         # gives text in the plot

yy  <- matrix(y,8,4)                   # converts y into a matrix (8*4)

est <- matrix(predict(model2),8,4)     # predict the estimates of model2(in 8*4 matrix)
windows()                              # opens a new window
day <- 0:3                             # day = 0,1,2,3

matplot(day,t(yy),type='p',xlim=c(0,4),ylab     =     'Chla     (microgram     /     li-
ter)',main='Exp1(Station 125, week 24)')             # gives plot of day and responses

matlines(day,t(est))                   # fits lines for the estimates

text(jitter(rep(3,4),3)+.4,est[1:4,4],c('C','P','N','PN'))
                                    # jitter separates the overlapped treatment names;
                                     # text gives the names of the estimates
```

Appendix 2

A. Summary of the significant coefficient regression of chlorophyll-a concentration measurement

Exp_2 Variables	Coeff	std. Error	t-value	p-value	
Intercept	4.74875	0.03273	145.11	< 2e-16	***
P	0.51438	0.03273	15.718	1.47E-11	***
N	1.13938	0.03273	34.816	< 2e-16	***
t1	0.244	0.01464	16.672	5.72E-12	***
t2	-0.24813	0.03273	-7.582	7.52E-07	***
t3	-0.08887	0.01464	-6.073	1.24E-05	***
P:N	0.53	0.03273	16.195	9.12E-12	***
P:t1	0.16762	0.01464	11.453	2.05E-09	***
N:t1	0.38012	0.01464	25.973	4.03E-15	***
P:t2	-0.1	0.03273	-3.056	0.00715	**
N:t2	-0.12375	0.03273	-3.781	0.00149	**
P:t3	-0.086	0.01464	-5.876	1.83E-05	***
N:t3	-0.11225	0.01464	-7.67	6.45E-07	***
P:N:t1	0.18275	0.01464	12.487	5.46E-10	***
P:N:t3	-0.09638	0.01464	-6.585	4.63E-06	***

Residual standard error: 0.1851 on 17 degrees of freedom
Multiple R-squared: 0.9947, Adjusted R-squared: 0.9904
F-statistic: 229.7 on 14 and 17 DF, p-value: < 2.2e-16
P-value of lack of fit F test: 0.04069649

Exp_3 Variables	Coeff	Std. Error	t-value	p-value	
Intercept	6.64781	0.07417	89.624	< 2e-16	***
P	0.18219	0.07417	2.456	0.0224	*
N	2.12969	0.07417	28.712	< 2e-16	***
t1	0.25369	0.03317	7.648	1.24E-07	***
t2	-0.20219	0.07417	-2.726	0.01234	*
t3	-0.09169	0.03317	-2.764	0.01132	*
P:N	0.22406	0.07417	3.021	0.00628	**
N:t1	0.61106	0.03317	18.421	7.38E-15	***
N:t2	-0.42156	0.07417	-5.683	1.03E-05	***
N:t3	-0.09381	0.03317	-2.828	0.00979	**

Residual standard error: 0.4196 on 22 degrees of freedom
Multiple R-squared: 0.9833, Adjusted R-squared: 0.9764
F-statistic: 143.6 on 9 and 22 DF, p-value: < 2.2e-16
P-value of lack of fit F test : 0.02954388

Exp_4 Variables	Coeff	Std. Error	t-value	p-value	
Intercept	8.22188	0.07055	116.54	< 2e-16	***
P	0.17	0.07055	2.41	0.02522	*
N	0.81563	0.07055	11.561	1.44E-10	***
t1	0.81912	0.03155	25.963	< 2e-16	***
t2	0.23688	0.07055	3.358	0.00298	**
P:N	0.18875	0.07055	2.675	0.01416	*
P:t1	0.0925	0.03155	2.932	0.00797	**
N:t1	0.37138	0.03155	11.771	1.04E-10	***
N:t2	0.23437	0.07055	3.322	0.00324	**
P:N:t1	0.12875	0.03155	4.081	0.00054	***
P:N:t2	0.18625	0.07055	2.64	0.01531	*

Residual standard error: 0.5617 on 25 degrees of freedom
Multiple R-squared: 0.9521, Adjusted R-squared: 0.9406
F-statistic: 82.88 on 6 and 25 DF, p-value: 2.829e-15
p-value of lack of fit F test: 0.01167196

Exp_5 Varibles	Coeff	Std. Error	t value	p-value	
Intercept	5.84656	0.05104	114.553	< 2e-16	***
P	0.15094	0.05104	2.957	0.007278	**
N	0.59906	0.05104	11.738	6.07E-11	***
t1	0.41944	0.02282	18.376	7.76E-15	***
t2	0.19031	0.05104	-3.729	0.001166	**
P:N	0.20344	0.05104	3.986	0.000624	***
P:t1	0.06131	0.02282	2.686	0.013489	*
N:t1	0.23519	0.02282	10.304	6.98E-10	***
N:t2	0.14344	0.05104	2.81	0.010192	*
P:N:t1	0.07956	0.02282	3.486	0.002094	**

Residual standard error: 0.2887 on 22 degrees of freedom
Multiple R-squared: 0.9671, Adjusted R-squared: 0.9537
F-statistic: 71.94 on 9 and 22 DF, p-value: 3.129e-14
p-value of lack of fit F test: 0.1741946

Exp_6 Variables	Coeff	Std. Error	t-value	p-value	
Intercept	6.34312	0.1389	45.666	<2e16	***
N	0.60813	0.1389	4.378	0.00015	***
t1	0.187	0.06212	3.01	0.00548	**
N:t1	0.24375	0.06212	3.924	0.00052	***

Residual standard error: 0.7857 on 28 degrees of freedom
Multiple R-squared: 0.6091, Adjusted R-squared: 0.5672
F-statistic: 14.54 on 3 and 28 DF, p-value: 6.72e-06
p-value of lack of fit F test : 0.879984

Exp_7 Variables	Coeff	Std. Error	t-value	P-value	
Intercept	6.94719	0.07226	96.145	<2e-16	***
N	1.03781	0.07226	14.363	7.09E-14	***
t1	0.35681	0.03231	11.042	2.59E-11	***
t2	-0.27969	0.07226	-3.871	0.000654	***
P:N	-0.22031	0.07226	-3.049	0.005224	**
N:t1	0.41719	0.03231	12.91	8.15E-13	***

Residual standard error: 0.4088 on 26 degrees of freedom
Multiple R-squared: 0.9523, Adjusted R-squared: 0.9431
F-statistic: 103.8 on 5 and 26 DF, p-value: 2.496e-16
p-value of lack of fit F test: 0.2473154

Exp_8 Variables	Coeff	Std. Error	t-value	p-value	
Intercept	8.77531	0.0608	144.341	<2e-16	***
P	0.16406	0.0608	2.699	0.01207	*
N	1.16406	0.0608	19.147	<2e-16	***
t1	0.45694	0.02719	16.806	1.75E-15	***
P:t1	0.07794	0.02719	2.867	0.00812	**
N:t1	0.42419	0.02719	15.602	1.02E-14	***

Residual standard error: 0.3202 on 25 degrees of freedom
Multiple R-squared: 0.9768, Adjusted R-squared: 0.9712
F-statistic: 175.4 on 6 and 25 DF, p-value: < 2.2e-16
p-value of lack of fit F test: 0.1012584

Exp_9 Variables	Coeff	Std. Error	t value	P-value	
Intercept	8.26219	0.09138	90.414	<2e-16	***
P	0.26156	0.09138	2.862	0.008586	**
N	1.43281	0.09138	15.679	4.12E-14	***
t1	0.21869	0.04087	5.351	1.71E-05	***
P:N	0.37469	0.09138	4.1	0.000409	***
P:t1	0.10981	0.04087	2.687	0.012883	*
N:t1	0.45431	0.04087	11.117	5.98E-11	***
P:N:t1	0.15644	0.04087	3.828	0.000813	***

Residual standard error: 0.5169 on 24 degrees of freedom
Multiple R-squared: 0.9488, Adjusted R-squared: 0.9339
F-statistic: 63.56 on 7 and 24 DF, p-value: 6.018e-14
p-value of lack of fit F test: 0.1989238

Exp_10 Variables	Coeff	Std. Error	t-value	p-value	
Intercept	5.46375	0.04340	125.901	< 2e-16	***
P	0.21000	0.04340	4.839	8.77e-05	***
N	1.47938	0.04340	34.089	< 2e-16	***
t1	0.08988	0.01941	4.631	0.000144	***
t3	-0.06463	0.01941	-3.330	0.003180	**
P:N	0.21313	0.04340	4.911	7.40e-05	***
P:t1	0.05563	0.01941	2.866	0.009247	**
N:t1	0.39900	0.01941	20.559	2.16e-15	***
N:t2	-0.32062	0.04340	-7.388	2.88e-07	***
N:t3	-0.06825	0.01941	-3.517	0.002050	**
P:N:t1	0.08175	0.01941	4.212	0.000391	***

Residual standard error: 0.2455 on 21 degrees of freedom
Multiple R-squared: 0.9882, Adjusted R-squared: 0.9826
F-statistic: 175.8 on 10 and 21 DF, p-value: < 2.2e-16
p-value of lack of fit F test: 0.3103994

Exp_11 Variables	Coeff	Std. Error	t value	p-value	
Intercept	4.22891	0.08556	49.424	< 2e-16	***
P	0.45797	0.08556	5.352	1.95e-05	***
N	0.98516	0.08556	11.514	5.02e-11	***
t3	-0.16041	0.03827	-4.192	0.000349	***
P:N	0.42359	0.08556	4.951	5.27e-05	***
P:t1	0.13634	0.03827	3.563	0.001653	**
N:t1	0.27528	0.03827	7.194	2.52e-07	***
N:t2	-0.23859	0.08556	-2.788	0.010443	*
P:N:t1	0.14272	0.03827	3.730	0.001098	**

Residual standard error: 0.484 on 23 degrees of freedom
Multiple R-squared: 0.9264, Adjusted R-squared: 0.9008
F-statistic: 36.18 on 8 and 23 DF, p-value: 3.086e-11
p-value of lack of fit F test: 0.0966996

Exp_12 Variables	Coeff	Std. Error	t-value	p-value	
Intercept	4.33555	0.08400	51.615	<2e-16	***
P	0.58367	0.08400	6.949	3.48e-07	***
N	0.82930	0.08400	9.873	6.31e-10	***
P:N	0.57242	0.08400	6.815	4.77e-07	***
P:t1	0.19342	0.03756	5.149	2.85e-05	***
N:t1	0.20505	0.03756	5.458	1.31e-05	***
N:t2	-0.20070	0.08400	-2.389	0.0251	*
P:N:t1	0.21167	0.03756	5.635	8.42e-06	***

Residual standard error: 0.4752 on 24 degrees of freedom
Multiple R-squared: 0.9226, Adjusted R-squared: 0.9
F-statistic: 40.85 on 7 and 24 DF, p-value: 8.113e-12
p-value of lack of fit F test: 0.00845033

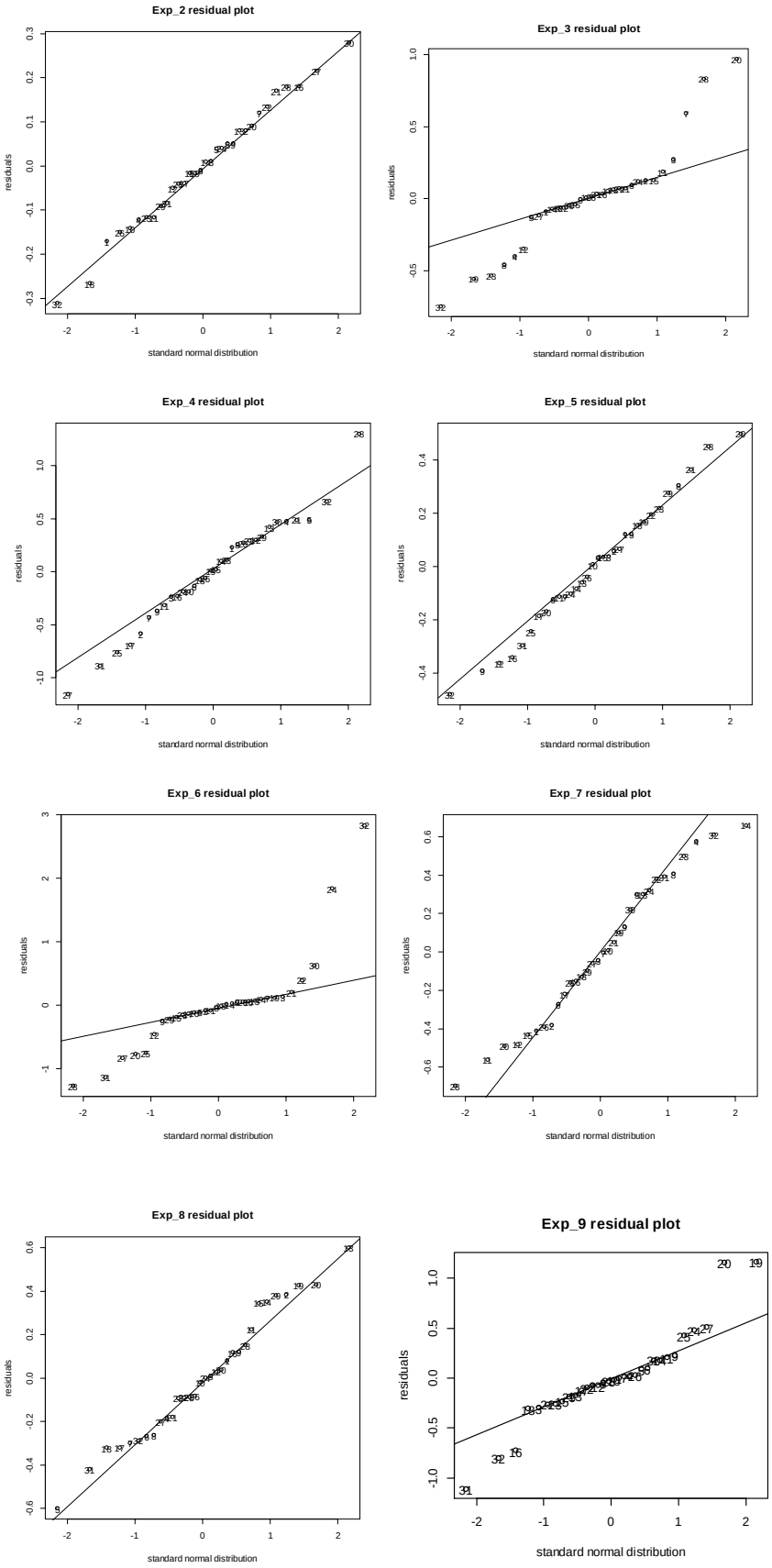
Exp_13 Variables	Coeff	Std. Error	t-value	p-value	
Intercept	7.42375	0.11643	63.761	<2e-16	***
P	0.53250	0.11643	4.574	0.000135	***
N	1.77000	0.11643	15.202	1.73e-13	***
t1	-0.23300	0.05207	-4.475	0.000172	***
P:N	0.54500	0.11643	4.681	0.000103	***
P:t1	0.13675	0.05207	2.626	0.015092	*
N:t1	0.47525	0.05207	9.127	4.16e-09	***
N:t2	-0.35250	0.11643	-3.028	0.005989	**
P:N:t1	0.18975	0.05207	3.644	0.001355	**

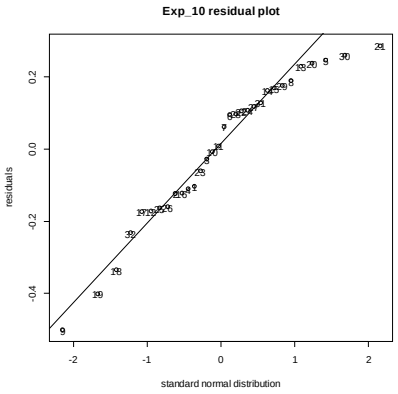
Residual standard error: 0.6586 on 23 degrees of freedom
Multiple R-squared: 0.9465, Adjusted R-squared: 0.9278
F-statistic: 50.83 on 8 and 23 DF, p-value: 8.398e-13
p-value of lack of fit F test: 0.1603053

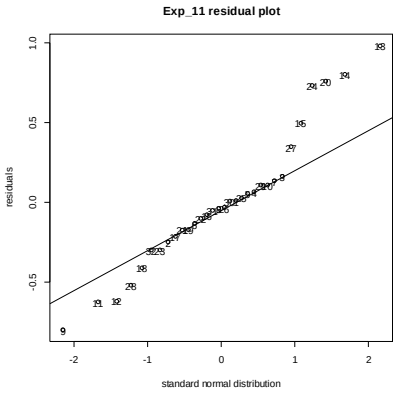
Exp_14 Variables	Coeff	Std. Error	t-value	p-value	
Intercept	8.40281	0.05994	140.178	<2e-16	***
P	0.52656	0.05994	8.784	2.67e-08	***
N	1.36844	0.05994	22.829	8.52e-16	***
t1	-0.10681	0.02681	-3.984	0.000730	***
t2	-0.32156	0.05994	-5.364	2.99e-05	***
P:N	0.67219	0.05994	11.214	4.46e-10	***
P:t1	0.11619	0.02681	4.334	0.000322	***
N:t1	0.36981	0.02681	13.795	1.12e-11	***
P:t2	-0.15781	0.05994	-2.633	0.015956	*
N:t2	-0.29844	0.05994	-4.979	7.22e-05	***
N:t3	0.07931	0.02681	2.959	0.007766	**
P:N:t1	0.16581	0.02681	6.185	4.83e-06	***

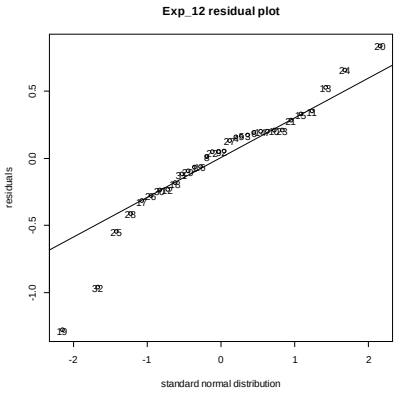
Residual standard error: 0.3391 on 20 degrees of freedom
Multiple R-squared: 0.9814, Adjusted R-squared: 0.9712
F-statistic: 96.05 on 11 and 20 DF, p-value: 7.998e-15
p-value of lack of fit F test: 0.03324718

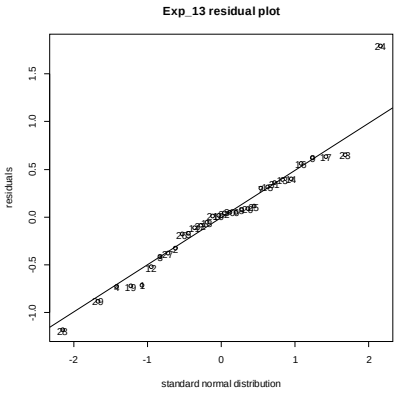
B. Normal probabilty plots of the Residuals of chlorophyll-a concentration

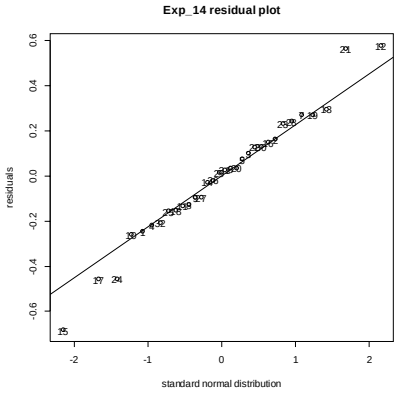



Exp_10 residual plot
residuals
standard normal distribution


Exp_11 residual plot
residuals
standard normal distribution


Exp_12 residual plot
residuals
standard normal distribution


Exp_13 residual plot
residuals
standard normal distribution


Exp_14 residual plot
residuals
standard normal distribution